The role of STAT5 and KRAS in hematopoiesis and acute myeloid leukemia

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The role of STAT5 and KRAS in hematopoiesis and acute myeloid leukemia

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Chapter 1

General introduction

1.1 Hematopoiesis

Hematopoiesis includes the formation and development of blood cells which is initiated by a small fraction of multipotent hematopoietic stem cells (HSCs). HSCs are located in the bone marrow and they have a capability for self-renewal. This means that after cell division at least one of the daughter cells will possess the same HSC characteristics as the mother cell.[1] The self-renewal ability of the HSCs maintains the stem cell pool and supplies cells for multilineage hematopoiesis during the entire lifespan of the individual.[2;3] During the process of hematopoiesis HSCs differentiate whereby long term-HSCs (LT-HSC) give rise to short term-HSCs (ST-HSC) which are then further differentiated into progenitor cells. Hematopoietic progenitors still have a strong proliferation potential but once they commit to certain lineages their proliferative capacity is reduced. Common myeloid progenitors (CMP) give rise to granulocyte–macrophage progenitors (GMP) which in turn produce monocytes, macrophages and granulocytes, and megakaryocyte-erythroid progenitors (MEP) which differentiate into megakaryocytes, platelets and erythrocytes.[4] Furthermore, ST-HSCs can differentiate into the lymphoid lineage giving rise to common lymphoid progenitors (CLP) and differentiate into mature T and B cells (Figure 1).[5]
Hematopoiesis is highly controlled by intrinsic mechanisms such as the expression of transcription factors or epigenetic modifications, and extrinsic mechanisms like growth factors, cytokines and the microenvironment or stem cell niche.[6] The balance between these mechanisms determines whether cells remain quiescent, proliferate, differentiate, self-renew, or undergo apoptosis.[7-9] In normal conditions, the majority of HSCs are

quiescent and mainly the more committed progenitors are proliferating and produce mature blood cells.[10]

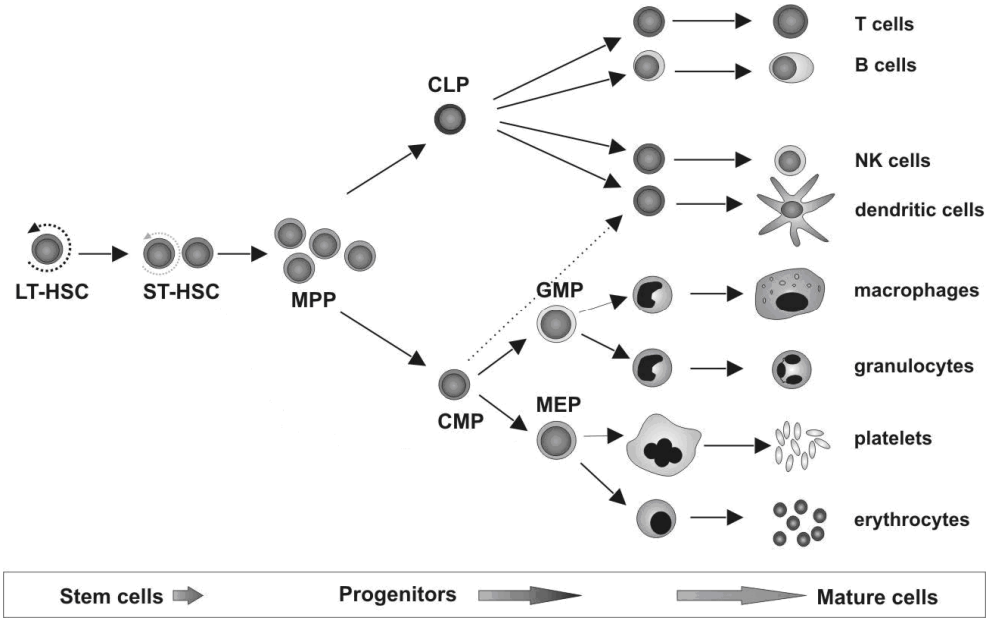

Figure 1. Hematopoiesis. Hierarchical representation of human and mouse hematopoiesis that shows the development of mature blood cells from hematopoietic stem cells. LT-HSC: long-term hematopoietic stem cell; ST-HSC: short-term hematopoietic stem cell; MPP: multipotent progenitor; CMP: common myeloid progenitor; CLP: common lymphoid progenitor; GMP: granulocyte/macrophage progenitor; MEP: megakaryocyte-erythroid progenitor.

1.2 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a clonal malignant disease which is characterized by rapid growth and accumulation of immature hematopoietic cells in the bone marrow causing disruption of normal hematopoiesis.[11;12] Leukemogenesis is a multistep process in which multiple genetic events following each other and ultimately induce the full leukemic phenotype.[12-14] For clinical purposes AML is classified according to World Health Organization (WHO) criteria predominantly based on morphology, chromosomal and molecular markers.[15] The WHO categories for AML classification are described below and summarized in Table 1.

AML with recurrent genetic abnormalities

This group includes AMLs with specific and recurrent gene mutations including AMLs with balanced translocations/inversions. During the chromosome rearrangements a fusion gene is formed which encodes a chimeric protein. This chimeric protein is required but usually not sufficient for leukemogenesis. The most commonly identified abnormalities are: t(8;21)(q22;q22), inv(16)(p13.1q22); t(16;16)(p13.1q22) and t(15;17)(q22;q21).

AML with myelodysplasia-related changes

This category of AML appears mainly in elderly patients and it is rare in children. There are three possible subtypes: AML preceded by MDS, AML with a MDS-related chromosomal abnormalities and AML with multilineage dysplasia. Frequent chromosome abnormalities include gain or

loss of major segments of chromosomes like : -5, -7/del(7q), +8, +9, +11, del(11q), del(12p), del(17p), -18, +19, del(20q), +21.

Therapy related myeloid neoplasms

This category includes those AML patients who developed AML after chemotherapy or radiation therapy as complication. Morphologically and genetically they resemble AML with myelodysplasia-related changes; a special subtype presents with mutations involving chromosome 11q23, the MLL gene.

AML not otherwise specified

Those AML cases are categorized in the group that does not fit in the previous categories. The basis for classification here is the morphological and cytochemical categorization of the blasts including the determination of the degree of maturation.

Table 1. WHO classification of acute myeloid leukemia and related neoplasms.

Name	Description	% of AML cases
Acute myeloid leukemia with recurrent genetic abnormalities	AML with t(8;21)(q22;q22), RUNX1-RUNX1T1	6
	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;p22); CBFB-MYH11	5-8
	Acute promyelocytic leukaemia with t(15;17)(q22;q12);PML-RARA	5-8
	AML with t(9;11)(p22;q23)MLLT3-MLL	2
	AML with t(6:9)(p23;q34); DEK-NUP214	0.7-1.8
	AML with inv(3)(q21q26.2) or t(3.3)(q21;q26.2); RPN1-EVl1	1-2
	AML (megakaryoblastic) with t(1:22)(p13;q13); RBM15-MKL1	<1
	AML with mutated NPM1	27-35
	AML with mutated CEBPA	6-15
Acute myeloid leukemia with myelodysplasia-related changes		24-35
Therapy-related myeloid neoplasms		10-20
Acute myeloid leukaemia not otherwise specified	AML with minimal differentiation	<5
	AML without maturation	5-10
	AML with maturation	10
	Acute myelomonocytic leukaemia	5-10
	Acute monoblastic and monocytic leukaemia	<5
	Acute erythroid leukaemia	<5
	Acute megakaryoblastic leukaemia	<5
	Acute basophilic leukaemia	<1
	Acute panmyelosis with myelofibrosis	rare

Based on the observed chromosomal abnormality AMLs can be categorized in separate risk groups including good, intermediate and poor-risk groups. Patients with abnormalities of t(15;17), or PML-RARα fusion gene, t(8;21), or AML-ETO fusion gene, and inv(16), or MYH11/CBFβ fusion gene, belong to the good-risk group. In the poor risk group complex cytogenetic abnormalities are frequently observed or monosomies of chromososmes 5 or 7. In addition AMLs with abnormalities of the long arm of chromosome 3, del(5q), del(7q), t(6;9), t(9;22) belong to unfavorable-risk group. The intermediate risk group has normal karyotype or karyotypes that are not described in the good or poor risk groups including +8, +6, del(12p), abn11q23, +21, and +22. This risk group can be further divided based on molecular abnormalities. Patients with mutation of the NPM1 gene without internal tandem duplications (ITDs) of the FLT3 receptor (FLT3-ITD) or bialleleic CEBPα mutation without FLT3-ITD mutation have a good prognosis, but patients without NPM abnormality will reside in the intermediate risk group.

1.3 Origin of the leukemic stem cells

The first demonstration of human leukemic stem cell (LSC) was published by Lapidot et al.[16] They showed that purified $CD34^+/CD38^-$ cells from human AML can be transplanted into NOD/SCID mice in most of the cases in contrast to $CD34^+/CD38^+$ or $CD34^-$ cells that did not engraft. Later, Bonnet and Dick observed that the malignant clone is hierarchically organized similar to the normal hematopoietic system where the

$CD34^+/CD38^-$ cells are higher in the hierarchy than the $CD34^+/CD38^+$ cells and that the frequency of the tumor initiating cell was approximately 1 per million AML blasts, establishing that very few AML cells had LSC capacity.[17]

However, the view that LSC reside selectively in the $CD34^+/CD38^-$ population was recently challenged by Taussig at al.[18] who demonstrated that anti-CD38 antibodies have an inhibitory effect on engraftment of cord blood (CB) cells as well as $CD38^+$ AML cells. When this inhibitory effect is blocked, the $CD34^+/CD38^+$ fraction can engraft from certain AML samples, although future studies will be needed to gain further insight into the LSC frequency within the $CD34^+/CD38^+$ population. Furthermore, it was recently suggested that in AMLs with the NPM mutation, the $CD34^-$ compartment might also contain LSC activity.[19]

These data show that there are only minor populations of leukemia-initiating cells that are capable of tumor initiation. LSCs reside on the top of the hierarchy of malignant cells similar to HSCs in normal hematopoiesis.[17;20-22]

There are many similarities between HSCs and LSCs; however an increasing amount of evidence suggests that LSCs not necessarily need to be derived from HSCs. It has been demonstrated that more committed progenitor cells can regain self-renewal potential. One of the examples is acute promyelocytic leukemia (APL). In more than 95% of the APL cases a retinoic acid receptor alpha (PML-RARα) fusion protein is detected which results from the t(15;17)(q22;q21) translocation. The progenitor origin of the disease is supported by the fact that in APL patients the PML-RARα expression is absent in the HSC ($CD34^+CD38^-$) compartment and $CD34^+CD38^-$ cells isolated from these patients did not engraft in NOD/SCID mice.[17;23]

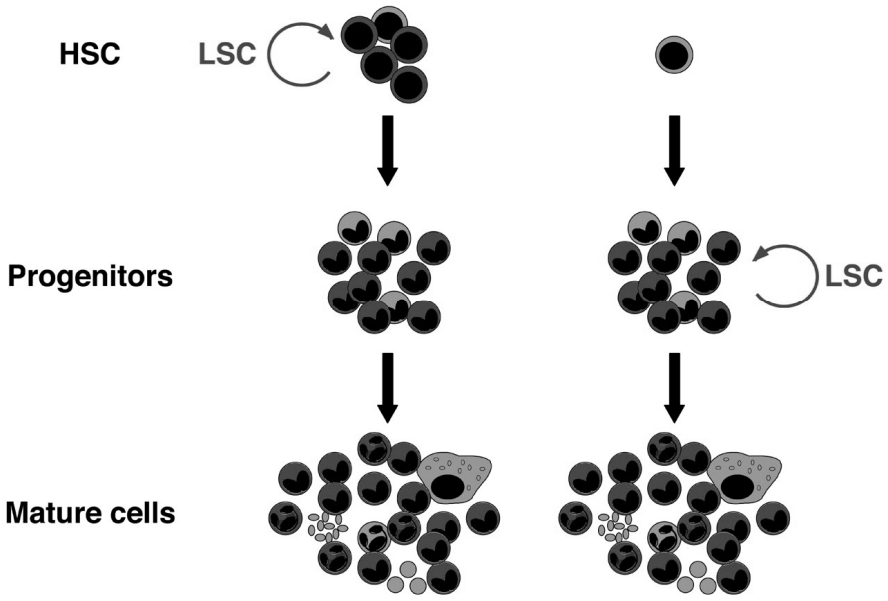

Figure 2. Models of stem cell leukemogenesis. Hematopoietic stem cells or more differentiated progenitors can be potential targets for primary and secondary mutations leading to acute myeloid leukemia. Adapted from Passegué et al.,[24]

Taking together, the tumor stem cell can arise from a stem cell or, in some special situations from the committed progenitor which is converted into a self-renewing leukemic stem cell (Figure 2).

1.4 Self-renewal and growth in leukemia

In normal hematopoiesis the balance between self-renewal, quiescence, growth and differentiation is very tightly controlled by intrinsic and extrinsic factors. A number of genes have been identified to be relevant for self-renewal and differentiation in the leukemic compartment but only a selection of them will be discussed in this chapter. The polycomb group proteins are extensively studied including BMI1. It has been demonstrated that BMI1 influences self-renewal of hematopoietic cells. Overexpression of BMI1 in cord blood $CD34^+$ cells increases self-renewal and induces symmetrical cell division in a mouse model system.[25-27] Furthermore, high BMI1 expression is a sign of unfavorable prognosis in AML and CML patients.[28;29] Downmodulation of BMI1 in human cord blood $CD34^+$ cells induced apoptosis and impairs self-renewal[27] while *Bmi1*$^{-/-}$ mice showed reduced competitive repopulation capacity.[26]

Overexpression of *EZH2,* another member of the polycomb group, has been linked to poor prognosis in prostate and breast cancer.[30;31] Kamminga et al. showed that overexpression of *Ezh2* in a mouse transplantation model prevents exhaustion of HSCs and it conserved long-term repopulating potential.[32] Furthermore, when EZH2 was inhibited in AML cell lines and primary AML cells it induced apoptosis indicating that EZH2 plays an important role in the survival of AML cells.[33]

Additional genes that have been identified as key players in leukemic transformation are the homeobox (*HOX*) genes. *HOX* genes are encoding transcription factors and they are organized in four clusters (A, B, C, and D) which are located on four different chromosomes. HOX proteins are key

regulators of the mammalian body plan and also important regulators of hematopoiesis. Several genes from *HOXA* and *HOXB* clusters are expressed in hematopoietic precursors[34] and during commitment they are downregulated.[35]

Animal experiments showed that mice overexpressing Hoxa9 had increased numbers of HSCs and myeloid progenitor cells and later they developed AML.[36;37] Overexpression of Hoxb4 in mice increased HSC self-renewal but did not change differentiation.[38] In addition, abnormal HOX gene expression is observed in a significant number of acute myeloid leukemia cases[39] and high expression of HOXA9 has been shown to be a negative prognostic parameter.[40] The highest HOX expression has been observed in a subset of patients with the NPM mutation.[41;42] Moreover, MLL fusion proteins dysregulate HOX and MEIS1 expression which is necessary for leukemic transformation.[43]

Chromosomal translocations involving HOX genes have been observed as well. Probably the most studied is the NUP98-HOXA9 fusion protein which arises from the t(7;11)(p15;p15) chromosomal translocation. This fusion protein acts as an aberrant transcription factor and its overexpression induces a preleukemic myeloproliferative disorder and later leukemia in mice.[44]

1.5 Signal transduction pathways and transcription factors in hematopoiesis and leukemia

1.5.1 The JAK-STAT pathway

The JAK-STAT pathway plays an important role in the cytokine-mediated signaling in human hematopoietic cells leading to various cellular functions, including self-renewal, proliferation, differentiation and apoptosis.[45-48] In general the binding of cytokines and growth factors, for instance GM-CSF, G-CSF, IL-3, IL-6, TPO, or EPO, to their receptors induces the multimerization of receptor subunits. Next, Janus kinases (JAK) are recruited and the receptor become activated by trans-phosphorylation which provides docking sites to SRC-homology 2 (SH2) domain-containing proteins. Important downstream targets which are recruited in the cytoplasmic domain of the phosphorylated cytokine receptor are the STAT proteins. Until now seven STAT family members have been identified, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. After phosphorylation STAT dimerizes and translocates to the nucleus where it alters gene transcription by binding to consensus DNA sequences (5'-TT(N_{4-6})AA-3').[49-52]

Suppressor of cytokine signaling (SOCS) proteins[53] and protein inhibitors of activated STAT (PIAS) are playing a crucial role in the regulation of STAT activation. SOCS proteins directly bind to JAKs and inactivate those[53] while PIAS binds to the phosphorylated STAT dimers preventing them from binding to DNA (Figure 3).[54]

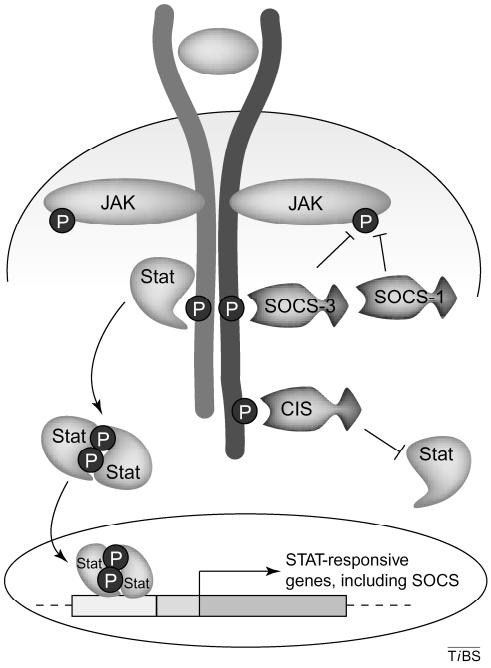

Figure 3. JAK-STAT signal transduction. The binding of cytokines and growth factors activates JAK, which phosphorylates the receptor and STAT proteins on specific tyrosine residues. STATs then dimerize, translocate to the nucleus, bind to specific the DNA sequence and initiate the transcription of target genes. SOCS proteins are members of the negative feedback loop which downregulate signal transduction from STAT pathway by inactivating JAKs. Adapted from Kile et al.[55]

Constitutive STAT5 activation in cord blood $CD34^+$ cells increases proliferation, self-renewal and enhances the interaction between the microenvironment and the hematopoietic cells.[56] Furthermore, when STAT5 was downmodulated in cord blood $CD34^+$ cells expansion and LTC-IC frequencies were reduced.[57] In AML blasts constitutive activation of STAT5 is frequently found due to activating mutations in upstream kinases such as FLT3 or c-KIT or due to the autocrine production of growth factors such as

GM-CSF.[58;59] *JAK2* mutations have been demonstrated in myeloproliferatieve disorders that result in constitutive STAT5 activation[60] while BCR-ABL-mediated activation of STAT5 is observed in chronic myeloid leukemia.[61]

The ability of STAT5 to induce a leukemic phenotype seems to correlate with STAT5 tetramer formation. Mice with increased levels of STAT5 tetramers developed disease but mutant STAT5 which did not form tetramers failed to induce leukemia.[62] In addition human leukemic samples showed increased STAT5 tetramer formation. These data suggest that enhanced STAT5 tetramer formation is essential for STAT5 induced leukemic phenotype.

There are many STAT5 target genes described which can explain these phenotypic changes. Increased STAT5 activation turns on cell cycle regulator genes like CYCLIN D1, CYCLIN D2, CYCLIN D3, p21; anti apoptotic genes like BCL-2, BCL-XL; growth promoting genes like PIM1 and genes that enhance cell adhesion like MUCIN1.

1.5.2 RAS signaling

RAS is a small (21 kDa) GTPase which functions as a molecular switch that controls intracellular signaling networks. There are three members of the RAS subfamily, H-RAS, K-RAS, and N-RAS. These isoforms have high homology within the first 185 amino-acids. The last 24 amino-acids contain the hypervariable region where specific posttranslational modifications occur.

RAS proteins contain a guanosine triphosphate (GTP) binding motif and cycle between the active GTP-bound and inactive GDP-bound forms. When a growth factor binds to its receptor the cytoplasmic tyrosine kinase domain becomes active and leads to phosphorylation of tyrosine residues which provide docking site for proteins possessing SH2 domains including GRB2. GRB2 recruit SOS and releases GDP from RAS. Next, RAS and GTP binds and the activated RAS further activates multiple signaling pathways (Figure 4) including the PI3K – AKT pathway, ERK and JNK pathway. One of the most important effectors of activated RAS is serine/threonine kinase RAF1. Upon activation RAF1 phosphorylates MEK1 and MEK2 which activate ERK1 and ERK2. Next, the phosphorylated ERK1 and ERK2 translocate to the nucleus where they activate ELK1, a nuclear transcription factor. ELK1 forms a complex with serum response factor (SRF) and activates numerous mitogen-inducible genes.

However, GTP-bound RAS can lead to the activation of other mitogen-activated protein kinases (MAPKs) for example p38 MAP kinase which phosphorylates and activates MAPKAP kinase 2 and activates transcription factors such as ATF2, MAC and MEF2.[63] Activation of the p38 pathway can negatively regulate cell cycle progression in the G1/S transition by downmodulating CYCLIN D1 or upregulating $p16^{INK4a}$ and induce differentiation by activating MEF2, or facilitate cell cycle exit.[64-67] In addition, p38 has tumor promotive functions that mediate cancer progression in part by modulating inflammation, migration and angiogenesis.[68-70]

Ras mutations are widely present in solid tumors and approximately in 30% of the AML cases.[71] The most frequently mutated sites are codon 12, 13, and 61 and these mutations result in constitutive activation of RAS.

However, there are other mechanisms that can induce RAS activation. Deregulation of upstream targets of RAS can lead to abnormal RAS activation.[72] Loss-of-function of a non-receptor protein tyrosine, KOS1, which normally down-regulates RAS activity by disrupting the GRB2-SOS1 complex and act as a tumor suppressor, can result in enhanced RAS activity.[73]

Many model systems were successfully generated in the past to study the role of RAS proteins in the hematopoietic system. RAS knock out mouse models showed that KRAS gene function is essential for normal mouse development, especially for the hematopoietic and central nervous system, while the function of NRAS and HRAS is dispensable.[74;75]

When three RAS isoforms, *KRAS*, *HRAS* and *NRAS* were compared using bone marrow transplantation model it became evident that all of the three oncogenes had the potential to induce myeloid leukemia with different phenotypes. Overexpression of NRAS induced either a chronic myelomonocytic leukemia or AML-like disease in the transplanted mice while KRAS-transduced bone marrow cells initiated a chronic myelomonocytic leukemia-like disease. Animals transplanted with HRAS-transduced cells developed an AML-like disease similar to NRAS, but in the case of HRAS the invasiveness of the tumor was higher and the latency of the disease was shorter.[76] In a different mouse model conditional expression of the $KRAS^{G12D}$ mutant induced a fatal monocytic myeloproliferative disease which was similar to chronic myelomonocytic leukemia and juvenile myelomonocytic leukemia. In the HSC compartment elevated levels of pSTAT5, pERK and p70S6 were detected which was induced by $KRAS^{G12D}$.[77] Overexpression of $KRAS^{G12D}$ from its endogenous locus gave a strong proliferative advantage to murine hematopoietic stem and

progenitor cells and initiated T-lineage leukemia/lymphoma which was associated with secondary NOTCH1 mutations. Additional studies demonstrated that the $KRAS^{G12D}$ induced myeloproliferative disease was restricted to HSCs and cooperating mutations appear later, during cancer progression.[78]

$NRAS^{G13C}$ overexpression in human $CD34^{+}$ CB cells showed increased proliferation in stromal cocultures and myeloid differentiation. These cells showed increased bone marrow engraftment in NOD/SCID mice and higher number of myeloid cells.[79]

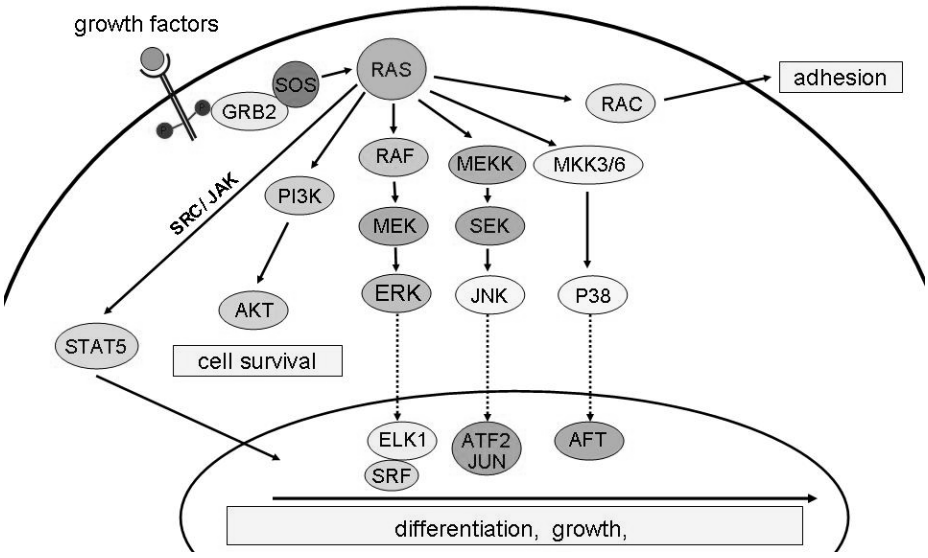

Figure 4. RAS signaling pathways. Activation of the growth factor receptor results in phosphorylation of key tyrosine residues which allow Grb2 to bind through their Src homology 2 (SH2) domain and this leads to the activation of downstream signaling cascades including RAS, AKT, ERK, STAT5, p38.

1.5.3 The role of reactive oxygen species in cell signaling and cancer

Increased reactive oxygen species (ROS) production is frequently observed in human malignancies including leukemia and it is often related to oncogene activation.[80-84] For example BCR-ABL overexpression increases ROS levels and activates AKT, GSK3β as well as β-Catenin which contribute to increased proliferation and high level of resistance to apoptosis.[85]

ROS are free radicals that derive from molecular oxygen. They can be taken up directly from the milieu by the cell or generated within the cell. There are various intracellular mechanisms which generate ROS for example aerobic metabolism, or it can be produced as second messenger in different signal transduction pathways by various enzymes like NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2.[86;87]

ROS molecules not only have a defense function as it was thought originally, but at low levels they play important roles in signal transduction. ROS can lead to conformational changes at the protein level which can diminish or promote DNA binding as well as change protein complex formation.[86;88-93] Increased ROS levels can stimulate the expression of c-FOS, c-JUN, and c-MYC mediated by PKC.[86;94-96] ROS can activate RAF1 and MEK1 through RAS and it can influence proliferation, apoptosis and differentiation. In addition, ROS activate NF-κB by releasing the inhibitory unit (IκB) from the NF-κB complex.[86;97;98] Moreover, ROS inhibit phosphatases which are necessary for turning off growth factor signaling by direct oxidation and disrupting normal cellular function.[99;100]

ROS influences long-term self-renewal of HSCs as well. In the study of Jang and Sharkis et al. it was demonstrated that HSCs can be sorted into two subpopulations based on ROS levels.[101] HSCs with low ROS levels represented the more primitive population and they had long-term repopulating capacity while HSCs with high ROS level were more differentiated and their engraftment was decreased. Since, ROS are highly reactive due to the presence of unpaired valence shell electrons they can cause damage to the cell if their level is too high. The most often caused harmful effects of high ROS level include the induction of DNA damage, lipid peroxidation, oxidations of amino acids in proteins and inactivating enzymes by oxidation.[102;103] In general cells have a defense mechanism to protect themselves against ROS damage by expressing enzymes such as superoxide dismutases, catalases, peroxidases and they also use cellular antioxidants such as flavenoids, ascorbic acid, tocopherol, glutathione or uric acid. This mechanism is necessary in order to counteract the negative effect of high ROS levels in the cell.[103]

In addition, FOXO family members seem to be important in the regulation of ROS levels by upregulating sets of genes which protect cells from ROS damage. In FOXO1/3/4 conditional knock out mice the short-term and long-term hematopoietic stem cell compartment was reduced and bone marrow transplantation experiments showed decreased long-term repopulating activity loss of quiescence, and increased cell cycle activity and apoptosis in the HSCs. This coincided with increased ROS levels in the HSC compartment and ROS induced gene expression changes. Furthermore, Thotova et al. showed that NAC treatment could reverse the FOXO-deficient HSC phenotype suggesting that FOXO decreased ROS levels in the HSC compartment and effected HSC survival and function.[104]

1.6 Bone marrow microenvironment and leukemia

It was proposed by Schofield et al. that HSCs locate in the bone marrow in a special "stem cell niche" which is crucial for maintaining stem cell characteristics.[105] The niche itself is quite complex and it contains various cell types as stromal cells, osteoblasts, mesenchymal cells, soluble factors (G-CSF, SCF, IL-6, SDF1)[106;107], extracellular matrix and blood vessels. These control HSC function and ultimately ensure a life-long reservoir of HSCs for hematopoiesis.

Until now, two types of niches have been described. One of them is the endosteal niche in which HSCs remain in close proximity to the osteoblasts. The other is the perivascular niche where the HSC is close to the vascular endothelium in the bone marrow.[108] Mouse model experiments showed that an increase in trabecular bone area correlated with increased numbers of LT-HSCs suggesting that osteoblasts are an important component of the niche and that they support HSCs.[109] Furthermore, increased parathyroid hormone (PTH) levels resulted in increased osteoblast numbers and in parallel the HSC numbers were increased as well.[110] These experiments show that the osteoblastic niche has the ability to keep the LT-HSCs in a quiescent stage and maintain LT-HSC numbers.[111]

Endothelial cells in the bone marrow have the ability to function as an alternative stem cell niche. However, there is increasing evidence that suggests that the vascular niche promotes proliferation as well as differentiation of HSCs and progenitor cells by providing a nutrient-rich microenvironment, high oxygen concentrations, and growth factors.[112;113]

Several molecular mechanisms have been reported that influence the interaction of the HSC with the niche. The association between the tyrosine kinase receptor TIE2 and its ligand angiopoietin-1 (ANG-1) is one of these mechanisms that enhances HSC quiescence.[111] Another important interaction for HSC–niche regulation is the interaction between SDF1 and CXCR4 which regulates the migration of HSC to the niche and influences the number of HSCs in the circulation.[114] In addition it has been described that HSCs express calcium sensing receptors that would direct them to the osteoblastic niche and that also mediate adhesion to the extracellular matrix protein collagen I.[115] Furthermore MUCIN1, a transmembrane glycoprotein, initiates a calcium-based signal transduction when it binds to intercellular adhesion molecule-1 (ICAM-1)[116] which is expressed on stromal cells.

The microenvironment for LSCs is not yet well understood. Prior studies have shown that certain oncogenes increase adhesion between LSCs and their microenvironment. For example overexpression of BCR-ABL in human hematopoietic cells increases adhesion by induction of focal adhesion-associated adaptor protein expression and increases the activation of RAC1.[117] However, AML1-ETO expressing CB $CD34^+$ cells can maintain their self-renewal capability and immature phenotype in liquid culture without stroma for more than 7 months suggesting that expression of AML1-ETO changes the microenvironment requirements of the transduced cells.[118]

In addition HIF (hypoxia-induced-factor) proteins may contribute to leukemic transformation in the stem cell niche. HIF is a transcriptional regulator which is assembled from an oxygen sensitive HIFα subunit (HIF1α, HIF2α or HIF3α) and a HIFβ subunit that is not oxygen sensitive

and is expressed constitutively. In normoxic conditions, the alpha subunit degrades while under hypoxia it is stabilized, binds to the HIFβ subunit and activates hypoxia-inducible genes by binding to hypoxia-responsive elements (HRE). This promotes neovascularization angiogenesis by vascular endothelial growth factor, glucose uptake by glucose transporter 1, and mitogenesis by activation of cMYC, OCT4, and p21. Especially the HIF2α isoform induces growth signaling and cell cycle progression through cMYC-dependent mechanisms.[119] It was suggested that HIF2α activation increases EGFR expression which contributes to tumor growth.[120] Furthermore, increased expression of HIF proteins is observed in the majority of solid tumors.[121] Since, HIFα proteins are stabilized under hypoxic conditions, as is observed in the osteoblastic niche, they may increase survival and contribute to maintenance of the LSCs.

Taken together, these studies show that disruption of genes responsible for the HSC-niche interaction might contribute to the development of leukemia and thereby influence the process of leukemic transformation.

1.7 Scope of this thesis

In this thesis we aimed to gain further insight in the mechanisms by which STAT5 and KRAS affect normal hematopoiesis and how they facilitate leukemic transformation. Constitutive activation of STAT5 and RAS has been shown in a number of AML cases. Although various model systems have been developed in order to study STAT5 and KRAS in normal hematopoiesis and leukemia, little information has become available on the molecular mechanisms that are involved. In **chapter two** we studied the role of a STAT5 target gene MUCIN1 and its ligand ICAM-1. Besides by STAT5, MUCIN1 is also upregulated by FLT3-ITDs and therefore the role of MUCIN1 was studied in normal hematopoiesis as well as in primary AML cells. Since, MUCIN1 is a transmembrane protein which is involved in adhesion and initiation of calcium signaling we aimed to elucidate its role in HSC self-renewal, maintenance and HSC–niche interactions. In **chapter three** we investigated whether STAT5 could impose long-term growth on HSCs specifically, or also on progenitor cells. We made use of a retroviral inducible model system in human CB $CD34^+$ cells in which STAT5 activation could be induced after treatment with 4-hydroxytamoxifen. We identified STAT5 target genes in specific stem cell and progenitor populations by microarray analysis. HIF2α was identified as one of the relevant STAT5 target genes which was studied further in detail. STAT5 fulfils important roles in erythropoiesis, but in megakaryopoiesis the potential role of STAT5 was less clear. In **chapter four** we investigated the effect of STAT5 on lineage commitment in MEPs (megakaryocyte/erythroid progenitors). We also performed gene expression profiling on STAT5

RNAi-transduced $CD34^+$ cells in order to gain further insight into megakaryocytic versus erythroid lineage fate decisions. In **chapter five** we studied the role of oncogenic KRAS in the process of self-renewal and differentiation. We analyzed whether KRAS as a single hit could result in long-term proliferation and we identified intrinsic and extrinsic mechanisms that are involved in the KRAS-induced phenotype. In **chapter six** the experimental work described in this thesis is summarized and discussed, and possible future directions are evaluated.

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Chapter 2

Mucin1 expression is enriched in the human stem cell fraction of cord blood and is upregulated in majority of the AML cases

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Abstract:

Mucin1 is a membrane glycoprotein that is overexpressed in a variety of human cancers. Here, we analyzed the role of Mucin1 in human hematopoietic stem/progenitor cells as well as in acute myeloid leukemia (AML) cells. Mucin1 expression was determined within the normal stem cell and progenitor compartment, as well as in the AML $CD34^+$ and $CD34^-$ subfractions of patient samples. Stem cells were enumerated in long-term culture-initiating cell (LTC-IC) assays in limiting dilution and progenitor frequencies in colony-forming cell (CFC) assays in methylcellulose, and consequences of elevated Mucin1 expression were studied using retroviral overexpression systems in cord blood (CB) $CD34^+$ cells. Ten percent of CB and 5% of peripheral blood $CD34^+$ cells expressed Mucin1. Retroviral overexpression of Mucin1 in CB $CD34^+$ cells resulted in elevated stem cell and progenitor frequencies as determined in LTC-IC and CFC assays without affecting differentiation, which coincided with increased proliferation. Overexpression of intercellular adhesion molecule-1, a ligand for Mucin1, in MS5 stromal cells further increased LTC-IC frequencies. Mucin1 overexpression was associated with increased nuclear factor-κB p50 nuclear translocation, suggesting that Mucin1-induced phenotypes involve increased cell survival mechanisms. Finally, we observed increased Mucin1 expression in 70% of the AML cases ($n = 24$), suggesting that elevated Mucin1 levels might be involved in regulating the proliferative potential of the immature leukemic compartment as well. Our data indicate that hematopoietic stem cells as well as $CD34^+$ AML subfractions are enriched for Mucin1 expression, and that overexpression of Mucin1 in CB cells is sufficient to increase both progenitor and LTC-IC frequencies.

Introduction

Mucin1 is a high molecular−weight glycoprotein expressed on most of the normal secretory epithelial cells. It is also present on nonepithelial cells, including B cells[1;2] and resting or activated T cells.[3] The full-length core protein of Mucin1 contains three domains. The human extracellular domain is built up from 20 amino acid tandem repeats that vary between 25 and 125.[4] This region of the protein can be extensively glycosylated because each of the tandem repeats contains five possible O-glycosylation sites. The highly conserved cytoplasmic tail interacts with various signal transduction molecules, including p53, β-catenin, Gsk3β, c-Src[5], and nuclear fact (NF)-κB.[6] The known ligands of Mucin1 are intercellular adhesion molecule-1 (ICAM-1)[7] and myelin-associated glycoprotein (MAG).[8] Upon activation the molecule is cleaved resulting in the formation of a cytoplasmic domain (Mucin1 CD) that can participate in signaling.[9;10] It was recently reported that in HCT116 colon cancer and HeLa cervical cancer cells Mucin1 constitutively activates NF-κB.[11] In colon carcinoma cells it has been demonstrated that Mucin1 alters activation of the phosphoinositide 3-kinase (PI3 K)/Pkb-Akt pathway and decreases Foxo3a phosphorylation[12], suggesting that Mucin1 protects against oxidative stress. Overexpression of Mucin1 is generally thought to promote metastasis by inhibiting cell adhesion.[13;14] However, other data suggest that Mucin1 may facilitate cell-to-cell adhesion as well by binding to ICAM-1, MAG, and E-selectin.[14] Classical transformation studies revealed that Mucin1 can be classified as an oncogene, as overexpression of Mucin1 was sufficient to induce colony formation in soft agar. Mucin1 overexpression has been observed in

hematological malignancies as well[15], although less is known about its function in human hematopoietic stem/progenitors cells and its potential role in the malignant transformation. Here, we describe that Mucin1 expression is enriched in the stem cell population within cord blood (CB) $CD34^+$ cells. Enforced overexpression of Mucin1 in CB $CD34^+$ cells using a retroviral approach resulted in enhanced long-term culture-initiating cell (LTC-IC) frequencies and elevated progenitor counts. In the leukemic counterpart elevated Mucin1 expression levels were observed in 70% of the investigated acute myeloid leukemia (AML) cases (n = 24).

Materials and methods

Cell culture and cell lines

CB $CD34^+$ cells were derived from neonatal cord blood from healthy full-term pregnancies after informed consent from the obstetrics departments of the Martini Hospital and University Medical Center in Groningen, The Netherlands and isolated by MiniMACS (Miltenyi Biotec, Amsterdam, The Netherlands) selection. For MS5 coculture and LTC-IC assays, cells were cultured in α−modified minimum essential media (Fisher Scientific Europe, Emergo, The Netherlands) supplemented with heat-inactivated 12.5% fetal calf serum (FCS), heat-inactivated 12.5% horse serum (HyClone Laboratories, Rodovre, Denmark), penicillin and streptomycin, 2 mM glutamine, 57.2 μM β-mercaptoethanol, and 1 μM hydrocortisone (Gartners medium). PG13 fibroblasts were grown in Dulbecco's modified Eagle's medium (Fisher Scientific Europe, Emergo, The Netherlands) supplemented with 10% FCS, penicillin and streptomycin, murine stromal cells (MS5)

were propagated in α−modified minimum essential medium supplemented with 10% FCS, penicillin, and streptomycin. Liquid culture experiments were performed in Iscove's modified Dulbecco's medium (PAA Laboratories CmbH, Cölbe Germany) supplemented with 10% FCS, 20 ng/mL interleukin (IL)-3, 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). TF1 cells were grown in RPMI-1640 medium (Fisher Scientific Europe) supplemented with heat-inactivated 10% FCS, penicillin, streptomycin, and 5 ng/mL recombinant human GM-CSF.

Colony-forming cell, LTC-IC, and cobblestone area–forming cell assays

Colony-forming cell (CFC) assays were performed as described[16;17], supplemented with 20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL G-CSF, 20 ng/mL c-kit ligand, and 6 U/mL erythropoietin. For secondary CFC assays, 2-week-old CFC cultures were harvested and half of the plate was replated in new CFC medium. After an additional 2 weeks, second CFCs were counted. LTC-IC limiting dilution assays were performed by plating (retrovirally transduced) CB $CD34^+$ cells on MS5 stromal cells in limiting dilutions in the range of 10 to 1000 cells per well in 96-well plates in Gartners medium. After 5 weeks, methylcellulose was added to the wells. Two weeks later, wells containing CFCs were scored as positive. Images were visualized using a Leica DM-IL microscope (Leica Microsystems, Rijswijk, The Netherlands) and a 40 × 0.60 numeric aperture objective.

Immunoblotting, histochemistry, and cytospins

Whole cell extracts were obtained by lysing 5×10^5 cells in boiling Laemmli sample buffer for 5 minutes prior to separation on 12% sodium dodecyl sulfate–acrylamide gels as described.[18;19] For Western blot

antibodies against Mucin1 (sc-7313), NF-κB (sc-298), Rb (sc-050), β-actin (sc-47778) were purchased from Santa Cruz Biotechnology Inc. (Tebu-Bio, Heerhugowaard, The Netherlands), anti-FLAG (F-3163) antibody from Sigma-Aldrich Corporation (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands), and anti–phospho-ERK1/2 (9106) from Cell Signaling Technology (Bioke, Leiden, The Netherlands). All primary antibodies for Western blot were used in 1:1000 dilutions. Secondary antibodies for Western blot were purchased from Dako Cytomation (Dako Cytomation, Glostrup, Denmark) and were used in 1:2000 dilutions. To isolate cytoplasmic and nuclear extracts 1×10^6 cells from tissue culture were collected, washed with 10 mL Tris-buffered saline (TBS) and pelleted by centrifugation at 1500*g* for 5 minutes. The pellet was resuspended in 1 mL TBS, transferred into an Eppendorf tube and pelleted again. TBS was removed and the cell pellet was resuspended in 400 µL cold buffer A (10 mM HEPES pH 7.9; 10 mM KC1; 0.1 mM ethylenediamine tetraacetic acid; 0.1 mM ethylene glycol tetraacetic acid; 1 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride) by gentle pipetting. Cells were allowed to swell on ice for 15 minutes, after which 25 µL 10% solution of Nonidet NP-40 from Fluka (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) was added and the tube was vigorously vortexed for 10 seconds. The homogenate was centrifuged for 30 seconds and the supernatant containing cytoplasm was transferred to a fresh tube and used as a cytoplasmic extract. The nuclear pellet was resuspended in 50 µL ice-cold buffer B (20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM ethylenediamine tetraacetic acid; 1 mM ethylene glycol tetraacetic acid; 1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride) and the tube was vigorously rocked at 4°C for 15 minutes on a shaking platform. The nuclear extract was centrifuged

for 5 minutes at 4°C and the supernatant was used as a nuclear extract. For histochemistry, cytospins were fixed in 4% paraformaldehyde, permeabilized in phosphate-buffered saline containing 0.1% Tween-20, and stained with antibodies in dilutions of 1:100. For histochemistry antibodies against Mucin1 (sc-15333) were purchased from Santa Cruz Biotechnology Inc. Secondary antibodies were purchased from Invitrogen and were used in 1:400 dilutions. May-Grunwald-Giemsa staining was used to analyze cytospins. Images were visualized using an Olympus BX50 microscope (Olympus Nederland, Zoeterwoude, The Netherlands) and a 63 × 1.3 numeric aperture oil objective.

Retroviral vectors, production, and transduction

For retroviral transduction the murine stem cell virus retroviral expression vector (MigR1 vector) was used, which contained an encephalomyelocarditis virus–derived internal ribosomal entry site (IRES2) in front of the enhanced green fluorescent protein (EGFP). Mucin1/pHβ Apr-1-neo-CR expression vectors were a kind gift from M.A. Hollingsworth.[20] The full-length Mucin 1 (Mucin1) and Mucin 1 in which the extracellular tandem repeat was deleted (DTR) were subcloned using the *Bam*H1 site of the Mucin1/pHβ Apr-1-neo-CR construct into the *Bgl*II site of the MigR1 vector. The pECFP-n1-ICAM-1 vector was a kind gift from Judith C. Hugh[21], which expresses a chimeric protein in which enhanced cyan fluoroscent protein is fused to the N-terminus of ICAM-1. ECFP – ICAM-1 was subcloned into the MigR1 retroviral vector in the *Xho*I/*Sal*I sites. All constructs were verified by sequencing. Stable retroviral producer cell lines were generated by transiently transfecting 293 T cells using FuGene-6 transfection reagent (Roche Diagnostics Nederland B.V.).

Transfection was performed as described previously.[22;23] $CD34^+$ cells were prestimulated for 48 hours in hematopoietic progenitor growth medium serum-free medium (Fisher Scientific Europe) supplemented with 100 ng/mL thrombopoietin, FLT3-ligand, and c-kit ligand. FLAG-tagged full-length Mucin1 as well as a Mucin1 mutant that lacks the extracellular tandem repeats (DTR) were stably expressed in CB $CD34^+$ cells together with EGFP as a marker gene using a retroviral approach. Appropriate overexpression of full-length Mucin1 (200 kDa) and truncated Mucin1 DTR (45 kDa) was verified by Western blot using FLAG-tag antibody. Stable TF1 MigR1, TF1 Mucin1, and TF1 DTR cell lines were created by transduction of TF1 cells. Retroviral supernatants were harvested from stable PG13 producers in HPGM for 8 to 12 hours. Before the first transduction round, supernatants were collected, and 4 μg/mL polybrene were added. Supernatants were filtered through 0.45-μm filters Corning Life Sciences (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and used for transduction of TF1 cells on retronectin-coated Takara Bio INC (Lucron Bioproducts B.V., Gennep, The Netherlands) 12-well plates. Three transduction rounds were performed and later transduced GFP-positive cells were sorted on a MoFlo (Dako Cytomation, Carpinteria, CA, USA). Mucin1 overexpression was checked by immunofluorescence staining. MS5 cells were transduced in three consecutive rounds using ICAM-1-ECFP PG13 producer cells lines similar as described here.

Quantitative polymerase chain reaction

For reverse transcriptase polymerase chain reaction (RT-PCR), total RNA was isolated from 1×10^6 cells using the RNeasy kit from QIAGEN (Venlo, The Netherlands) according to manufacturer's recommendations. RNA (200

ng) was used for reverse transcription with M-MuLV reverse transcriptase (Fermentas, St Leon-Roth, Germany) according to manufacturer's instructions as described previously.[24;25]

Flow cytometry analysis and cell sorting

All antibodies were obtained from BD Biosciences, except the Mucin1 antibody, which was purchased from Santa Cruz Biotechnology Inc. (catalogue number: sc-7313). Cells were incubated with antibodies at 4°C for 30 minutes and than washed once in phosphate-buffered saline. All fluorescein-activated cell sorting (FACS) analyses were performed on a FACS Calibur (Becton Dickinson) and data were analyzed using WinList 3D (Verity Software House, Topsham, ME, USA). Cell sorting of the $CD34^+$ cells into progenitor and HSC fractions was performed on the basis of the combinatorial expression of cell surface antigens. HSC were sorted as $CD34^+CD38^{low}$, common myeloid progenitors (CMP) as $CD34^+CD38^+CD123^+CD45RA^-$, granulocyte-macrophage progenitors (GMP) as $CD34^+CD38^+CD123^+CD45RA^+$ and megakaryocyte-erythroid progenitors (MEP) as $CD34^+CD38^+CD123^-CD45RA^-$. Sorting of cells was performed on a MoFlo (Dako Cytomation, Carpinteria, CA, USA).

Statistical analysis

Student's *t*-test was used to calculate the significance level between experimental groups. Data were expressed as mean ± standard error of the mean. p Values <0.05 were considered significant.

Results

$CD34^+/Mucin1^+$ fraction of human cord blood is enriched for HSC

As shown in Figure1A, approximately 10% of the CB $CD34^+$ cells expressed Mucin1 as determined by FACS analysis. LTC-IC assays were performed in limiting dilution on MS5 stroma with MoFlo-sorted populations, and the $CD34^+/Mucin1^+$ subpopulation was enriched twofold for LTC-ICs as compared to the $CD34^+/Mucin1^-$ subpopulation (Fig. 1B, average of n = 3 ± SD is: $Mucin1^-$: 1/2191 ± 214 vs $Mucin1^+$: 1/1140 ± 164, $p < 0.001$). $CD34^+/Mucin1^+$ and $CD34^+/Mucin1^-$ populations were propagated in long-term MS5 stromal cocultures and the strongest expansion was observed in cultures that were initiated with $CD34^+/Mucin1^+$-sorted cells (Fig. 1C). This coincided with strongly increased progenitor frequencies that were observed at weeks 1, 3, and 5 (Fig. 1D). To determine Mucin1 expression in HSCs and progenitor subpopulations CB $CD34^+$ cells were sorted using the following cell surface markers: $CD34^+/CD38^{low}$ HSC; $CD38^+CD34^+CD45RA^-CD123^+$ CMP; $CD38^+CD34^+CD45RA^+CD123^+$ GMP and $CD38^+CD34^+CD45RA^-CD123^-$ MEP. From each sorted group, 10^3 cells were plated in CFC assays in methylcellulose and colonies were evaluated after 2 weeks of culture, and MEP and GMP fractions were isolated at a purity of 95% (Fig. 1E). RNA was extracted from each sorted sample and Mucin1 expression was determined by quantitative PCR (Q-PCR). A threefold increase in Mucin1 expression was observed in the $CD34^+38^{low}$ HSC fraction as compared to the total $CD34^+$ control cells. Also, a 1.5-fold increase in Mucin1 expression was observed in the GMP fraction, while Mucin1 expression was twofold reduced in the MEP fraction as compared to CB $CD34^+$ control cells

(Fig. 1F). Mucin1 expression was measured at the protein level as well by FACS (Fig. 1 G). HSCs showed high Mucin1 expression (34%), which was lower in the common myeloid progenitors (19%) and MEP (6%) populations. Mucin1 expression was also observed in the GMP fraction.

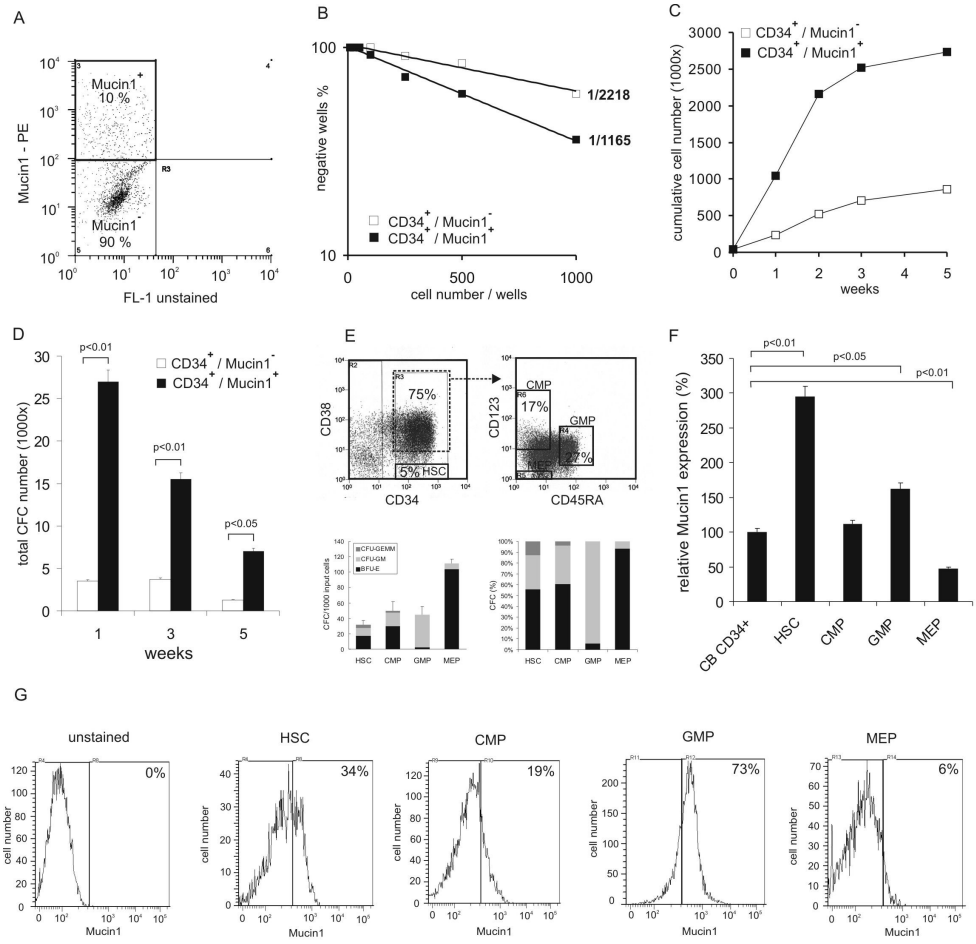


Figure 1. The CD34$^+$/Mucin1$^+$ fraction of human cord blood is enriched for hematopoietic stem cells. (A) A representative fluorescein-activated cell sorting (FACS) plot showing Mucin1 expression in cord blood (CB) CD34$^+$ cells. (B) CB CD34$^+$ cells were

sorted into Mucin1^{+} and Mucin1^{-} populations and stem cell frequencies were determined by long-term culture-initiating cell (LTC-IC) assays. (B) A representative dataset from three independent experiments is shown. (C) CB CD34^{+} cells were sorted into Mucin1^{+} and Mucin1^{-} populations, which were plated on MS5 stromal cells and cultures were weekly demi-depopulated. Weekly cumulative cell counts are shown. (D) Assay as in (C) and progenitor frequencies were determined in the harvested suspension fraction by colony-forming cell (CFC) assays in methylcellulose. (D) Shows the average of three independent experiments. (E) CB mononuclear cells were sorted into hematopoietic stem cells (HSC: CD34^{+}CD38^{low}), common myeloid progenitors (CMP: CD34^{+}CD38^{+}CD123^{+}CD45RA^{-}), granulocyte-macrophage progenitors (GMP: CD34^{+}CD38^{+}CD123^{+}CD45RA^{+}) and megakaryocyte-erythroid progenitors (MEP: CD34^{+}CD38^{+}CD123^{-} CD45RA^{-}). The purity of the sort was determined by CFC assays. (F) Quantitative polymerase chain reaction from sorted HSC, CMP, GMP, and MEP fractions shows that the Mucin1 mRNA expression is highest in the CD34^{+} /CD38^{low} fraction as compared to more differentiated progenitor subsets. (G) Mucin1 protein expression on HSC, CMP, GMP, and MEP fractions was measured by FACS. Data shows one out of three representative experiments.

Mucin1 is overexpressed in the majority of the AML cases

Mucin1 expression was analyzed in large number of AML samples (n = 24), CB (n = 7), and peripheral blood (n = 6). According to the French-American-British (FAB) classification the AML cases were classified as M0 (n = 2); M1 (n = 4); M2 (n = 2); M4 (n = 3), and M5 (n = 11). FLT3-internal tandem duplication mutations were observed in 20% of the cases (Table 1). Mucin1 expression was determined in the sorted CD34^{+} and CD34^{-} fractions of the AML samples both at the mRNA level by Q-PCR (Fig. 2A) and at the protein level by FACS (Fig. 2C) and good correlations were observed between Mucin1 expression at mRNA and protein level. Approximately 70% of the AML cases showed increased Mucin1 expression as compared to CD34^{+} control CB or PB cells (Fig. 2D). In

seven (30%) AML cases Mucin1 expression was particularly increased in the AML $CD34^+$ population, while in the other cases Mucin1 expression was increased in both the AML $CD34^+$ and $CD34^-$ populations (Fig. 2C and D). Mucin1 overexpression did not correlate with either FAB classification or other measured parameters including karyotype, risk group, or CD34 percentage in the mononuclear AML cell fraction (Table 1).

Table 1. Summary of patient details

						Fold-change in $Mucin1^+/CD34^+$ cells in AMLs compared to PB	
AML	FAB	FLT3-ITD	Karyotype	Risk group	% $CD34^+$ cells	by FACS	by Q-PCR
2003 119	M0	+	Normal	Intermediate	80	ND	4.6
2005 258	M0	ND	5q-, trisomy 6	Poor	90	ND	6.3
2003 160	M1	—	3p+,-7,8 p-	Poor	15	23.7	7.8
2005 024	M1	—	inv (3q), 7-, 10-	Poor	87	39.5	ND
2005 038	M1	—	Normal	Intermediate	2	0.5	ND
2003 117	M1	—	del(9)	Intermediate	86	ND	0.6
2005 051	M2	ND	Normal	Intermediate	37	20.0	ND
2005 107	M2	ND	t(8;21), t(q22;q22)	Intermediate	14	0.3	ND
2003 152	M4	—	inv(16)	Intermediate	43	46.2	29.7
2005 165	M4	—	Normal	Intermediate	7	ND	39.6
2005 062	M4	ND	Normal	Intermediate	69	9.0	ND
2006 004	M5	—	3q-, 5q+, 8+	Poor	8	1.5	2.2
2005 060	M5	—	9q-	Intermediate	1	17.6	ND
2004 019	M5	—	trisomy 9	Intermediate	67	8.9	ND
2005 049	M5	—	t(6;11), t(q27;q23)	Poor	20	12.2	ND
2004 182	M5	+	Normal	Intermediate	7	6.4	ND
2005 198	M5	+	Normal	Intermediate	1	10.0	ND
2005 145	M5	+	Normal	Poor	12	0.6	ND
2004 094	M5	—	Normal	Intermediate	5	0.5	ND
2005 289	M5	—	Normal	Intermediate	85	ND	6.3
2004 113	M5a	—	ND	Intermediate	2	0.5	ND
2003 114	M5B	+	t(11;20)	Intermediate	49	ND	1.1
2004 024	Sec	ND	Normal	Poor	89	11.8	ND
2003 120	Sec	ND	Complex including t(3;5),-5	Poor	8	ND	6.1

AML = acute myeloid leukemia (AML); FACS = fluorescein-activated cell sorting; ITD = internal tandem duplication; ND = not determined; PB = peripheral blood; Q-PCR = quantitative polymerase chain reaction; Sec = secondary AML.

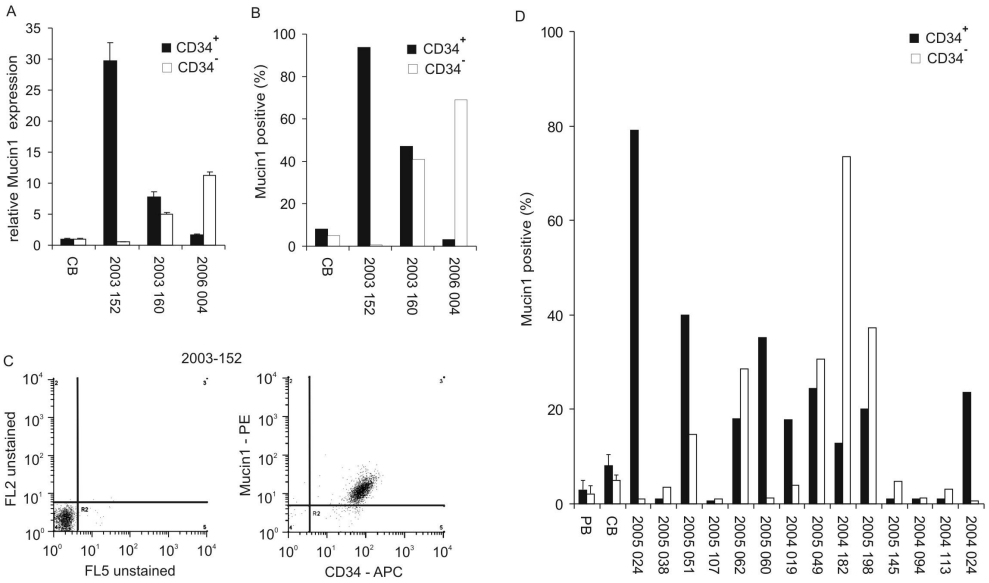


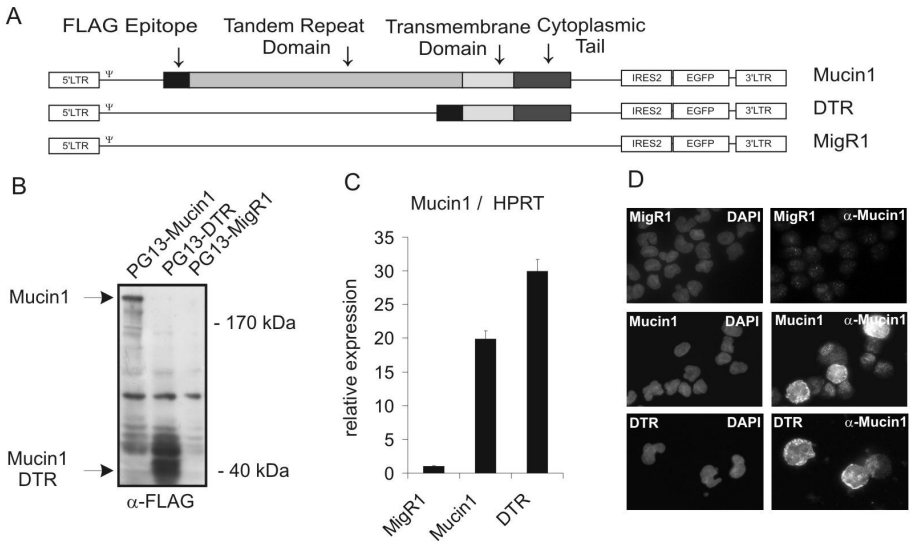
Figure 2. Mucin1 is overexpressed in the majority of the acute myeloid leukemia (AML) cases. (A) Cord blood (CB) and three AML samples were sorted into $CD34^+$ and $CD34^-$ fractions and Mucin1 mRNA expression levels were measured by Quantitative polymerase chain reaction. Error bars were calculated from triplicates. (B) Experiment as in (A) but now Mucin1 protein expression levels were determined by fluorescein-activated cell sorting (FACS) analysis. At least five samples were measured from CB and peripheral blood (PB) sample. (C) FACS data plots show expression of Mucin1 from one characteristic AML samples. Left panels are unstained controls. (D) Mucin1 protein expression levels were determined in the $CD34^+$ and $CD34^-$ fractions of 14 AML cases as determined by FACS analysis.

Enhanced proliferative capacity, LTC-IC frequencies and self-renewal of CB $CD34^+$ cells by overexpression of Mucin1

Because Mucin1 was highly expressed in AML $CD34^+$ cells, we wished to study whether overexpression of Mucin1 in normal $CD34^+$ cells would affect growth and self-renewal of stem and progenitor cells. FLAG-tagged

full-length Mucin1 as well as a Mucin1 mutant that lacks the extracellular tandem repeats (DTR) were stably expressed in CB $CD34^+$ cells together with EGFP as a marker gene using a retroviral approach (Fig. 3A). PG13 Virus producer cell lines were generated and Mucin1 expression levels were measured by Western-blot using FLAG-tag antibody, which revealed appropriate overexpression of full-length Mucin1 (200 kDa) and truncated Mucin1 DTR (45 kDa, Fig. 3B). Overexpression in transduced CB $CD34^+$ cells was confirmed by Q-PCR analysis (Fig. 3C). To confirm overexpression at the protein level, Mucin1 immunostaining on transduced CB $CD34^+$ cells was performed and demonstrated cytoplasmic localization of the protein (Fig. 3D). To determine the biological effects of Mucin1 overexpression CB $CD34^+$ cells were transduced with Mucin1 and DTR and cells were cultured in long-term MS5 cocultures for 5 weeks. Weekly demi-depopulations were performed that revealed a substantial increase (twofold) in expansion of both Mucin1 and DTR cultures as compared to the control (Fig. 3E). FACS analysis for CD11b, CD14, CD15, CD36, CD71, and glycophorin A revealed no differences in differentiation markers toward myeloid or erythroid cells as compared to controls (data not shown). Furthermore, expanded cells from long-term cocultures were analyzed for the presence of progenitors by performing CFC assays in methylcellulose. As depicted in Figure 3F and G, a substantial increase in progenitors was observed both in the Mucin1 as well as DTR cultures, as compared to MigR1 controls. No differences in the distribution between colony-forming unit granulocyte-macrophage, burst-forming unit erythroid, and colony-forming unit granulocyte-erythrocyte-macrophage-megakaryocyte colonies were observed (data not shown). Colonies from CFC assays were harvested and replated into new methylcellulose to determine the self-renewal

capacity of progenitors. Upon second plating, both Mucin1 and DTR groups gave rise to secondary colonies, which were not observed in the MigR1 control group (Fig. 3H). Cells from secondary replates, however, did not give rise to third CFCs (data not shown). To determine whether the increase in cell number in the cocultures are related to an increase in cell cycle or decrease in apoptosis, we performed cell-cycle analysis and Annexin-V staining. No significant changes among the different subgroups were observed (data not shown). Also, CyclinD1 expression was tested by Q-PCR, but no difference was found between the control, the Mucin1-, or DTR-transduced cells (data not shown).



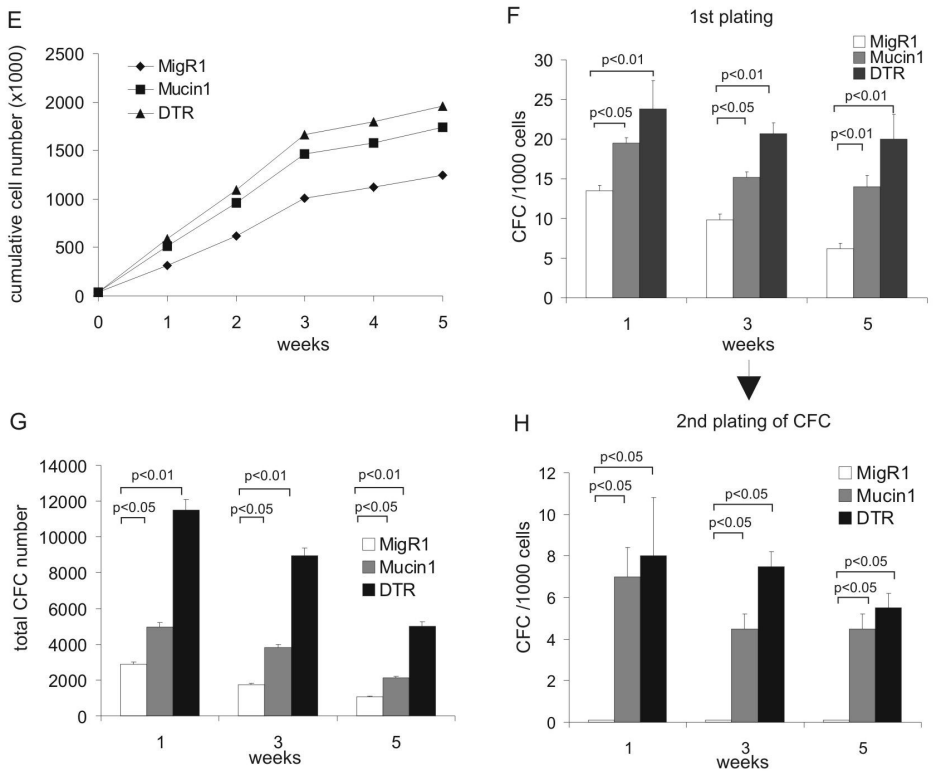


Figure 3. Enhanced proliferative capacity and self-renewal of cord blood (CB) CD34$^+$ progenitor cells by overexpression of Mucin1. (A) Schematical representation of retroviral vectors used in this study. (B) Overexpression of Mucin1 was determined from PG13 stable virus producer cell lines by Western blot using anti-FLAG antibody. (C) Quantitative polymerase chain reaction and (D) immunofluorescence staining shows overexpression of Mucin1 in transduced CB CD34$^+$ cells. (E) CB CD34$^+$ cells were transduced with Mucin1 isoforms (Mucin1 and DTR) and MigR1 control, cultures were weekly demi-depopulated, cell counts of suspension cells are indicated. (E) Representative dataset from three independent experiments. (F) Cells that were harvested by weekly demi-depopulation from MS5 coculture as described in (E) were analyzed for progenitor content by colony-forming cell (CFC) assays. The data shows the number of colonies arising from 1000 plated cells. (F) Average of three independent experiments. (G) Experiment as in (F), but now the total number of colony-forming cells in the cultures is indicated. (H) Cells from CFC assays shown in (F) were replated to a secondary CFC assay and arising colonies were evaluated after 2 weeks.

To determine whether Mucin1 expression increases stem cell frequencies we performed LTC-IC assays in limiting dilution. Cells expressing Mucin1 had an approximate twofold increase and DTR-transduced cells had an approximate threefold increase in stem cell frequency (Fig. 4A) (average of $n = 3 \pm SD$ is MigR1: $1/2227 \pm 116$ vs Mucin1: $1/1252 \pm 215$; $p = 0.01$; MigR1: $1/2227 \pm 116$ vs DTR: $1/836 \pm 97$; $p < 0.01$). Comparable results were obtained when LTC-IC assays that were performed in bulk in T25 flasks. After 5 weeks of coculture of DTR and Mucin1-transduced cells, significantly more and larger cobblestone formation was observed as compared to MigR1 controls. Methylcellulose was added to the MS5 cocultures at week 5 and after an additional 2 weeks LTC-ICs in bulk were enumerated. As shown in Figure 4B, LTC-IC frequencies were increased fourfold in the Mucin1 group (MigR1: 23 ± 3 vs Mucin1: 83 ± 4; $p = 0.006$), while in the DTR-transduced group a 10-fold increase (MigR1: 23 ± 3 vs DTR: 255 ± 7; $p < 0.001$) in LTC-IC number was observed as compared to controls. As ICAM-1 acts as a ligand for Mucin1 it was tested whether overexpression of ICAM-1 in MS5 would further increase LTC-IC frequencies. ICAM-1 − ECFP was stably overexpressed in MS5 stromal cells using a retroviral approach and overexpression of ICAM-1 in MS5 cells was measured by FACS (Fig. 4C). The functionality of ICAM-1 overexpression was determined by Western blotting using antibodies against phosphorylated ERK, which is a downstream target of ICAM-1 (Fig. 4D). LTC-IC frequencies were determined on control and ICAM-1–overexpressing MS5 with MigR1 and Mucin1-transduced CB cells. As shown in Figure 4E, the LTC-IC frequencies were increased significantly in the ICAM-1 MS5 cultures, both in control as well as Mucin1 overexpressing cells.

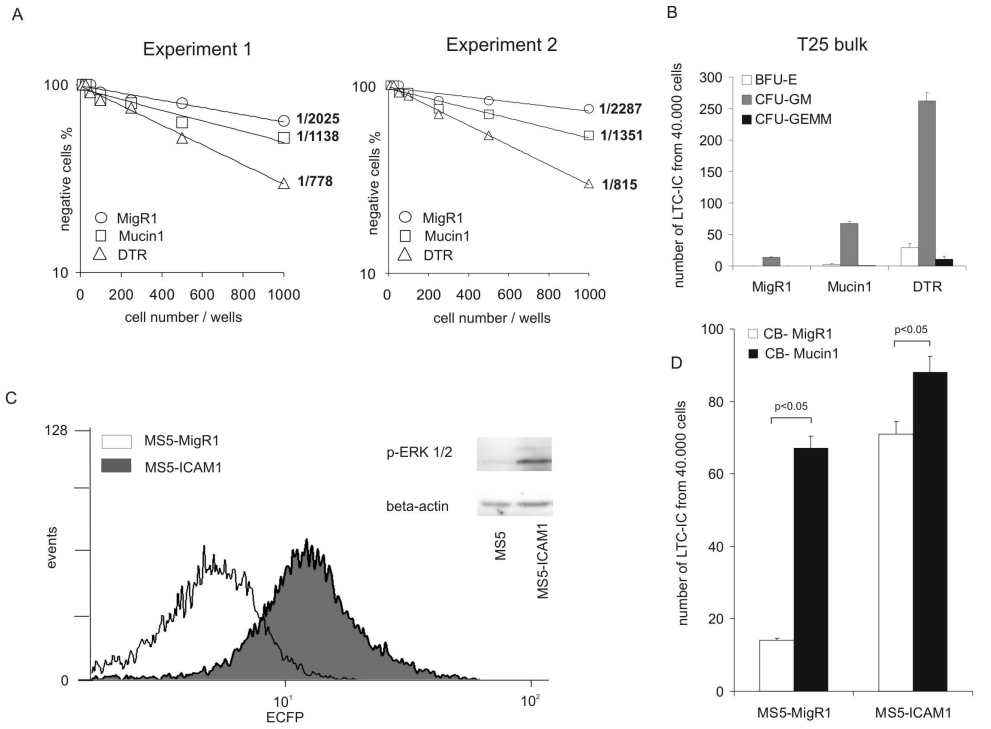


Figure 4. Enhanced long-term culture-initiating cell (LTC-IC) frequencies and by overexpression of Mucin1. (A) Cord blood (CB) $CD34^+$ cells were transduced with Mucin1, DTR, and MigR1 control. After transduction cells were sorted and LTC-IC assays in limiting dilution were performed on MS5 bone marrow stromal cells. Data shows the stem cell frequencies from two representative experiments from four independent experiments. (B) Stem cell frequencies were determined in bulk cultures by plating 40,000 cells on T25 flasks with MS5 stroma, cultures were weekly demi-depopulated and fed with new medium, and at week 5 methylcellulose was added to the flasks. Colonies were counted 2 weeks later. (C) MS5 stromal cells were transduced with retroviral vectors expressing the ECFP−intercellular adhesion molecule-1 (ICAM-1) fusion protein or MigR1 control vectors and cells were sorted by MoFlo. enhanced cyan fluorescent protein − ICAM-1 expression was determined by fluorescein-activated cell sorting (FACS) for enhanced cyan fluorescent protein. (D) The functionality of ICAM-1 overexpression was determined by Western blotting using antibodies against phosphorylated ERK1/2. (E) LTC-IC frequencies were determined from cocultures initiated with MigR1 and Mucin1

transduced CB cells and using MS5-MigR1 (control) and MS5-ICAM1 stroma. After 5 weeks of culture methylcellulose was added and colonies were counted 2 weeks later.

Long-term maintenance of progenitors expressing Mucin1 requires bone marrow stromal cells

To determine whether the above-mentioned phenotype on MS5 coculture is stroma-dependent, we cultured $CD34^+$ transduced CB cells in liquid culture in the presence or absence of cytokines (IL-3 and stem cell factor). In the absence of cytokines, all cells died within 7 days (data not shown). This suggests that Mucin1 overexpression does not overcome the cytokine-dependency of hematopoietic cells. In the presence of cytokines the Mucin1 and DTR-transduced groups showed 1.5-fold increases in cell numbers as compared to MigR1 controls, respectively (Fig. 5A). This increase in expansion might arise from an increase in progenitors that was observed in CFC assays in methylcellulose, particularly at day 7 and 18 (Fig. 5B and C). This increase in progenitors coincided with an increase in the percentage of $CD34^+$ cells, as determined by FACS analysis (Fig. 5D). However, the increase in progenitors and $CD34^+$ cells was transient and could not be maintained for >20 days, and at day 36 no $CD34^+$ cells or progenitors could be observed in liquid-culture conditions. FACS analysis for CD11b, CD14, CD15, CD36, CD71, and glycophorin revealed no differences in differentiation markers toward myeloid or erythroid cells as compared to controls (data not shown).

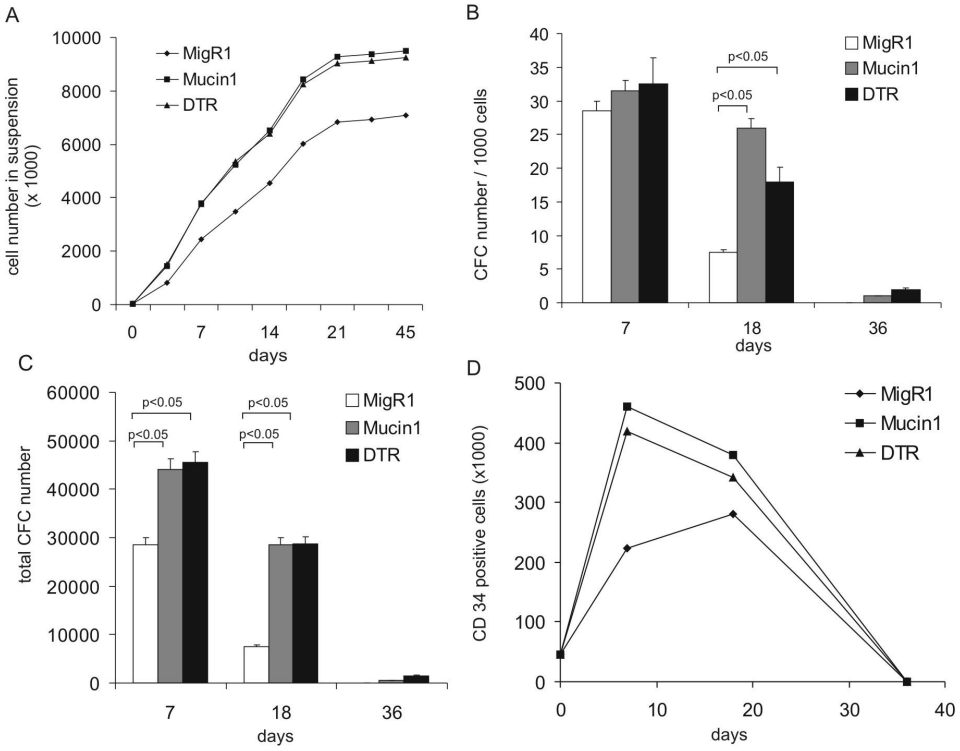


Figure 5. Mucin1 expressing progenitors can not be maintained long-term outside of a bone-marrow microenvironment. (A) Cord blood (CB) $CD34^+$ cells were transduced with Mucin1, DTR, and MigR1 control. Transduced CB cells were sorted and cultured in liquid culture in the presence of cytokines (interleukin-3 (IL-3) and granulocyte macrophage colony-stimulating factor (GM-CSF)). Expansion was monitored weekly and cumulative cell counts are shown. (B) Cells that were harvested weekly from liquid cultures as described in (A) were analyzed for progenitor content in methylcellulose assays. The data shows the number of colonies arising from 1000 plated cells. (C) As in (B), but now the total progenitor output per culture is indicated. (B) and (C) show the average of three independent experiments. (D) The percentage of $CD34^+$ cells in liquid cultures was determined by fluorescein-activated cell sorting analysis and the total number of $CD34^+$ cells in each culture is indicated.

Mucin1 overexpression results in increased NF-κB p50 nuclear translocation

To study which signal transduction pathways are affected by Mucin1 overexpression, we generated TF1 cell lines that were transduced with MigR1 control vector, Mucin1, or DTR. Mucin1 overexpression was checked by immunofluorescence staining (Fig. 6A). Proliferation of the transduced cell lines was determined in MS5 bone marrow stromal cocultures. Half medium change was performed two times a week and suspension cells were counted. Overexpression of Mucin1 and DRT resulted in a proliferative advantage relative to MigR1 control cells, comparable to expansion profiles of CB $CD34^+$ transduced cells (Fig. 6B). Cytoplasmic and nuclear fractions were prepared and were analyzed by Western blotting using antibodies against NF-κB and β-catenin. Our results showed a decrease in NF-κB p105 (uncleaved form) levels in the cytoplasmic fraction and increased NF-κB p50 (cleaved form) levels in the nuclear fraction upon either Mucin1 or DTR overexpression. The purity of nuclear and cytoplasmic extracts was determined by Western blotting for retinoblastoma. β-actin was used as a loading control (Fig. 6C). Western blot analysis for β-catenin did not show any alteration in either expression levels or nuclear accumulation of β-catenin in response to Mucin1 signaling in these hematopoietic cell lines (data not shown).

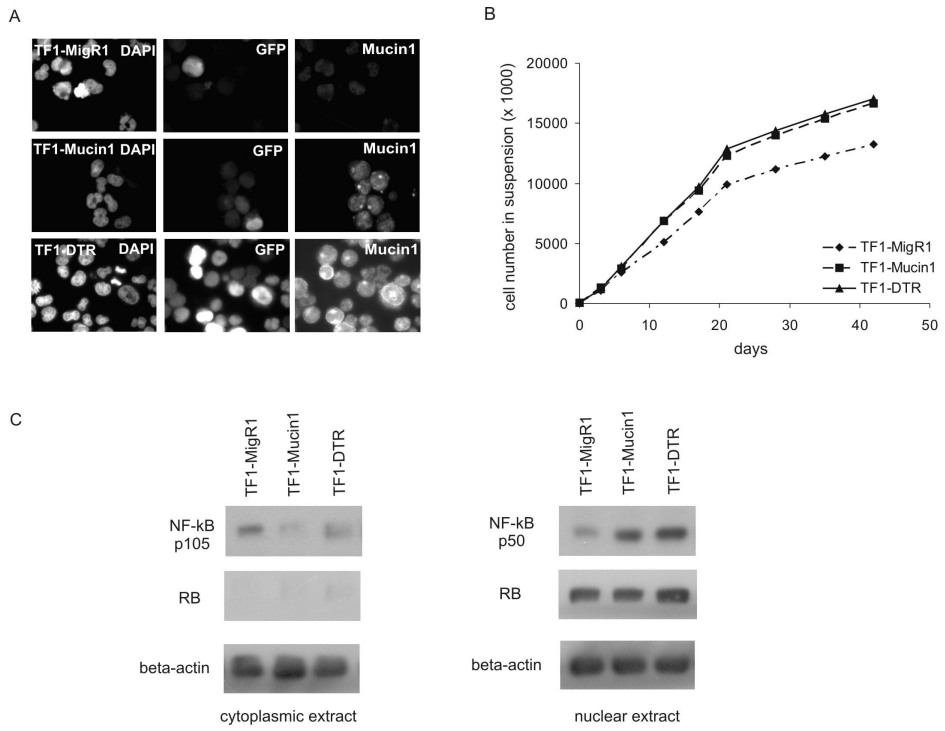


Figure 6. Mucin1 overexpression increases nuclear factor NF-κB nuclear translocation in TF1 cells. (A) TF cells were transduced with Mucin1, DTR, and MigR1 retroviral particles. Immunofluorescence staining shows overexpression of Mucin1 in transduced TF1 cells. (B) Cells were plated from each TF1 stable cell line in MS5 coculture and they were cultured for 6 weeks. Half medium change was performed two times a week, expansion was monitored and cumulative cell counts are shown. (C) Nuclear and cytoplasmic fractions were isolated from TF1-MigR1, TF1-Mucin1, and TF1-DTR cell lines. A Western blot analysis was performed using antibodies against p105 and p50. Purity of nuclear fractions was determined by reprobing the blots with antibodies against retinoblastoma.

Discussion

The studies described here identify Mucin1 as a novel transmembrane protein that is expressed on human hematopoietic stem and progenitor cells. Mucin1 is expressed predominantly in the HSC and GMP populations, and LTC-IC frequencies within the $CD34^+$ population were enriched twofold by comparing $Mucin1^+$ vs $Mucin1^-$ cells. Enforced expression of Mucin1 in CB $CD34^+$ cells elevated LTC-IC and progenitor frequencies, which coincided with a proliferative advantage of Mucin1-transduced cells. The majority of AML cases also displayed increased levels of Mucin1 in the $CD34^+$ subfraction, suggesting that both normal as well as leukemic stem and progenitor cells might utilize Mucin1 signaling as a mode to expand the immature hematopoietic compartment.

Our understanding of the mechanisms that determine whether, where and when a stem cell will self-renew or differentiate is still limited, but recent advances have indicated that the stem cell microenvironment, or niche, provides essential cues that direct these cell-fate decisions. Within the niche, a direct ligand for Mucin1, ICAM-1, is expressed on stromal cells. Upon activation, Mucin1 can be cleaved close to the membrane-proximal region, resulting in an intracellular Mucin1 protein that can associate with or activate various signaling pathway components.[26;27] It has been demonstrated that the cytoplasmic c-terminal domain of Mucin1 has a β-catenin binding site, which after cleavage can shuttle β-catenin into the nucleus where it alters gene transcription.[28] One of the known target genes is cyclinD1, which can contribute to enhanced proliferation. Also, we have extensively tested the possibility that Mucin1-induced β-catenin signaling is

involved in the enhanced proliferation of CB $CD34^+$ cells by determination of cytoplasmic and nuclear translocation of β-catenin using Western blotting, immunofluorescence microscopy, as well as TCF/LEF-driven luciferase reporter assays, but failed to demonstrate Mucin1-induced activation of β-catenin (data not shown). Neither did we observe increased CyclinD1 expression levels induced by Mucin1 by Q-PCR. Another signaling pathway that contributes to malignant transformation and can also be activated by Mucin1 overexpression is the NF-κB signaling pathway. Recently, it was published that overexpression of Mucin1 in human carcinoma cells is associated with constitutive activation of NF-κB p65 in epithelial cells.[29] In our TF1 model system, we could confirm that Mucin1 overexpression induces nuclear NF-κB translocation. These data suggest that the Mucin1-induced phenotypes might involve increased cell survival and/or a reduced susceptibility to apoptotic signals. Both the full-length wild-type Mucin1 and the DTR mutant that no longer has the capacity of binding to ligands such as ICAM-1 were able to activate NF-κB signaling. Thus, these data suggest that intracellular signaling is ultimately the main mode of action of Mucin1 in the human stem and progenitor cells. However, overexpression of ICAM-1 in the stromal cells did result in increased LTC-IC frequencies, which suggest that ICAM-1–mediated Mucin1 signaling might indeed facilitate an increased interaction between hematopoietic cells and stromal cells, resulting in a protective effect on stem/progenitor cells. Our data favor a model in which Mucin1 and DTR-induced NF-κB signaling enhance survival of HSCs, but these HSCs still require a bone marrow microenvironment for stem cell maintenance. Interaction of $Mucin1^+$ HSCs with their bone marrow microenvironment in which ICAM-1 is expressed results in activation of Mucin1, and possibly Mucin1

cleavage, generating intracellular Mucin1 isoforms that can participate in intracellular signaling such that NF-κB is activated.

Studies in the leukemic counterpart demonstrated that approximately 70% of the investigated AML cases displayed increased Mucin1 expression levels as compared to normal controls. No correlations were detected with treatment results, FAB classification, or karyotype. Increased expression levels were predominantly observed in the $CD34^+$ AML fraction, although Mucin1 expression was elevated in the $CD34^-$ fraction of some AMLs as well. Our observations are in line with previous publications in which Mucin1 expression was also observed in a variety of hematological malignancies, including AML.[30] Importantly, it was shown that Mucin1 can be recognized by and attract cytotoxic T lymphocytes and thus might be used for immunotherapeutic targeting in AML. In some more recent studies the glycosylation profile of Mucin1 was determined in AML and multiple myeloma cases. AML cells only expressed the fully glycosylated form of Mucin1, but not the cancer-associated form.[31;32] While we have not been able to determine the glycosylation status in our AML samples, these data suggest that a glycosylation-specific antibody-mediated immunotherapy in AML might not be feasible. Yet, we do find strongly increased Mucin1 expression levels as compared to normal controls, leaving open the possibility that targeting of $Mucin1^+$ AMLs using cytotoxic T-lymphocytes might have clinical applicability.

In the majority of AML cases, leukemic stem cells are retained within the $CD34^+$ compartment and we have recently been able to establish an ex vivo expansion assay in which long-term cultures could be established that resided exclusively within the AML $CD34^+$ fraction.[33] Thus, our observations appear to be in line with the possibility that elevated Mucin1

levels are involved in regulating the proliferative potential of the immature leukemic compartment as well. The promoter of Mucin1 contains a STAT binding element around −500 bp, and we have indeed been able to confirm that Mucin1 is upregulated by activating mutants of STAT3 and STAT5 in CB $CD34^+$ cells by performing microarray and Q-PCR analysis (unpublished observations S.F. and J.J.S.). Similarly, introduction of FLT3-internal tandem duplications also resulted in increased Mucin1 expression levels. As we and others have observed that signal transducer and activator of transcription (STAT) 3 and STAT5 are constitutively activated in a large number of AML cases.[34-36] Our data pinpoint to a model in which STAT-mediated upregulation of Mucin1 expression might contribute to the process of leukemic transformation in AML. Within our dataset, we did indeed observe that AMLs with internal tandem duplications in the FLT3 gene displayed high Mucin1 expression levels, although more AMLs need to be investigated to determine the statistical significance of these findings. Taken together, our data indicate that HSCs as well as AML $CD34^+$ cells are enriched for Mucin1 expression, and that overexpression of Mucin1 in CB cells is sufficient to increase both progenitor and LTC-IC frequencies.

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Chapter 3

Identification of HIF2α as a STAT5 target gene in human hematopoietic stem cells involved in self-renewal

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Summary

The transcription factor STAT5 fulfils essential roles in self-renewal in mouse and human HSCs and its persistent activation contributes to leukemic transformation, although little molecular insight has been obtained in the underlying mechanisms. Here, we describe that STAT5 can impose long-term expansion and self-renewal exclusively on human hematopoietic stem cells, but not on progenitors. This was associated with an enhanced interaction with the bone marrow microenvironment of STAT5-transduced HSCs. Using genome-wide gene expression profiling in human stem and progenitor subpopulations we identified 33 STAT5 target genes in HSCs. HIF2α was prominent upregulated by STAT5 in HSCs and was tested functionally using a lentiviral RNAi-approach. These experiments revealed that STAT5-induced long-term expansion and elevated HSC frequencies depended on HIF2α expression, while differentiation was not affected. Our studies indicate that pathways normally activated under hypoxia might be utilized by STAT5 under higher oxygen conditions to maintain and/or impose HSC self-renewal properties.

Introduction

Hematopoiesis is a dynamic process in which self-renewal, proliferation, lineage commitment, differentiation and apoptosis are tightly controlled. Signal transducer and activator of transcription 5 (STAT5) is a transcription factor that fulfils important roles in many of these processes.[1;2] STAT5 is widely expressed throughout the hematopoietic system, both in stem and progenitor cells as well as in committed erythroid, myeloid and lymphoid cells [1;3;4] and can be activated by different cytokines. In most cases, JAK tyrosine kinase activity mediates STAT5 tyrosine phosphorylation, although the tyrosine kinase receptor family can also induce STAT5 phosphorylation in a JAK-independent manner. After cytokine-induced phosphorylaton, STAT5 dimerizes and translocates to the nucleus where it induces expression of target genes.[4;5]

Loss-of-function experiments have revealed critical roles for STAT5 in the hematopoietic stem/progenitor compartment. STAT5AB$^{\Delta N/\Delta N}$ mice which express a truncated form of STAT5 have been used to assess stem cell function in the absence of wt STAT5 signaling. These mice were characterized by normal HSC numbers and stem cells isolated from the bone marrow or fetal liver were capable of engrafting irradiated recipients.[6] Yet, competitive repopulating capacity of STAT5AB$^{\Delta N/\Delta N}$ HSCs was severely impaired.[7-11] Experiments with STAT5AB$^{-/-}$ mice that completely lack expression of STAT5 revealed that STAT5 was required for the development of HSCs, lymphocytes and erythrocytes, while myelopoiesis was not affected in these animals.[12] It was demonstrated recently by Wang et al. that conditional deletion of STAT5 in a Mx1-Cre mouse model

decreases the HSC pool size, survival and the quiescence of the HSCs indicating a role of STAT5 in the maintenance of HSC quiescence under physiological conditions.[13] In human CB $CD34^+$ cells downmodulation of STAT5 resulted in decreased CFC numbers and LTC-IC frequencies which coincided with a reduction in long-term expansion, while cell differentiation was not affected.[14]

The most direct evidence for STAT5 acting as an oncogene arises from murine BM transplantation studies in which constitutively activated STAT5 (S711F) mutants were overexpressed. Lethally irradiated recipients receiving activated STAT5-transduced BM died within 6 weeks after transplantion of a multilineage leukemia.[15] It was demonstrated that a tryptophan residue in the N-terminal region of STAT5 is required for tetramerization of STAT5 dimers, and tetramer-deficient STAT5 mutants were unable to induce leukemia in mice.[16] Another activating mutant of STAT5, STAT5A(1*6) that contains 2 point mutations (H299R and S711F) [17] was also shown to induce a myeloproliferatieve disorder.[18] But only when the most primitive $CD34^-$LSK population was transduced.[19] In contrast, human stem and progenitor cells overexpressing constitutively activated STAT5A imposed long term self-renewal but without malignant transformation.[20] However, in the majority of acute myeloid leukemia (AML) cases constitutive activation of STAT5 has been observed.[21-23] This might be a characteristic feature of upstream activated kinases such as FLT3, KIT or JAK2 [24-26] or due to autocrine growth factor production.[27] Targeting STAT5 in primary AML $CD34^+$ cells resulted in impaired long-term expansion and a decline in the formation of leukemic CAFCs.[28]

Although it is clear that STAT5 fulfils essential roles in both normal hematopoiesis as well as in the development or maintenance of leukemia,

little is known about the molecular mechanisms that are involved. Our current data show that STAT5 can impose a long-term proliferative advantage on the $CD34^+/CD38^-$ HSC population, but not on progenitors. Gene expression profiling in STAT5-transduced HSC and progenitor cells as well as in STAT5 activated GATA1 downmodulated cells led to identification of 33 GATA1 independent STAT5 target genes in the HSC population that could be linked to the STAT5-induced long-term proliferation and self-renewal phenotype. One of the identified genes was Hypoxia Induced Factor 2α (HIF2α/EPAS1) and functional studies revealed that STAT5-induced expansion of HSCs and enhanced progenitor and stem cell frequencies were mediated, at least in part, via HIF2α upregulation, while differentiation was not affected.

Materials and methods

Cell cultures and cell lines

From healthy full-term pregnancies neonatal CB was obtained after informed consent from the obstetrics departments of the University Medical Center Groningen (UMCG) and in Martini Hospital Groningen, The Netherlands. After separation of mononuclear cells by Lymphocyte Separation Medium (PAA Laboratories, Coble, Germany), CB $CD34^+$ cells were isolated by Mini-MACS Separation System (Miltenyi Biotec, Amsterdam, The Netherlands). Cells for MS5 coculture and LTC-IC assays were cultured in α-modified medium essential media (Fisher Scientific Europe, Emergo, The Netherlands) supplemented with heat-inactivated 12.5% fetal calf serum (Lonza, Leusden, The Netherlands) and heat-

inactivated 12.5% horse serum (Invitrogen, Breda, The Netherlands), penicillin and streptomycin (all from PAA Laboratories), 57.2 μM β-mercaptoethanol (Merck Sharp & Dohme BV, Haarlem, The Netherlands) 1 μM hydrocortisone (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands), designated as Gartners medium.

Colony-forming cell and LTC-IC assays

Colony-forming cell (CFC) assays were performed as it was described previously.[29] Retroviraly transduced $CD34^+$ CB cells were used for LTC-IC limiting dilution assays in the range of 5-1000 cells per well in a 96 well plate using Gartners medium. Methylcellulose (StemCell Technologies, Grenoble, France) supplemented with 20 ng/mL IL-3, 20 ng/mL IL-6 (both of them from Gist-Brocades, Delft, The Netherlands), 20 ng/mL G-CSF (Rhone-Poulenc Rorer, Amstelveen, The Netherlands), 20 ng/mL c-kit ligand (Amgen, Thousand Oaks, USA), and 6 U/mL erythropoietin was added at week five and two weeks later wells containing CFCs were scored as positive and the LTC-IC frequency was calculated by ELDA.[30]

Retroviral / lentiviral production and transduction

For lentiviral transduction we used a pLVUT lentiviral vector where the short hairpin against human HIF2α (GCTGACTCTTTGCTCTAAT) was cloned into the ClaI EcoR1 restriction site. For control a scrambled shRNA was used. Viral particles for lentiviral transduction were produced by cotransfection of 293T cells with 0.7 μg pcDNA3-VSVg-REV, 3 μg pCMV D8.91, and 3 μg pLVUT-scrambled RNAi or pLVUT-HIF2-RNAi. Lentiviral supernatants were collected after 24 hours and they were stored at

–80°C. Stable PG13 STAT5A(wt)-ER [31] retroviral producers were cultured in DMEM (Lonza, Leusden, The Netherlands) supplemented with heat-inactivated 10 % fetal calf serum and penicillin/streptomycin (PAA Laboratories). For retroviral transduction viral particles were harvested from PG13 cultures after 8-12 hours of incubation in HPGM. Supernatants were collected before the transduction rounds; were filtered through 0.45-mm filters (Millipore B.V., Amsterdam Zuidoost, The Netherlands). CB $CD34^+$ cells were cultured for 48 hours in hematopoietic progenitor cell growth medium (HPGM) supplemented with stem cell factor (SCF; 100 ng/mL), Flt3 ligand (Flt3L; 100 ng/mL; both from Amgen, Thousand Oaks, USA), and thrombopoietin (TPO; 100 ng/mL; Kirin, Japan) and transduced on retronectin-coated plates (Lucron Bioproducts B.V, Gennep, The Netherlands) in 3 consecutive rounds of 8 and 12 hours with lentiviral or retroviral supernatant supplemented with the same cytokines and 4 μg/mL polybrene. After transduction, transduced GFP-positive, NGFR-positive or double positive cells were sorted on a MoFlo (Dako Cytomation, Carpinteria, CA, USA).

Flow cytometry analysis and cell sorting

Antibodies used for FACS analysis and for cell sorting were the following: CD34, CD38, CD45RA, CD123, CD15, CD71 CD235a (BD Bioscience, Breda The Netherlands) and CD 11b, CD14 (BD Bioscience, Breda The Netherlands). Cell sorting of HSC and progenitor fractions from $CD34^+$ CB cells was performed on the basis of the combinatorial expression of cell surface antigens. HSC were determined as $CD34^+CD38^{low}$, common myeloid progenitors (CMP) as $CD34^+CD38^+CD123^+CD45RA^-$, granulocyte-macrophage progenitors (GMP) as

$CD34^+CD38^+CD123^+CD45RA^+$ and megakaryocyte-erythroid progenitors (MEP) as $CD34^+CD38^+CD\ 123^-CD45RA^-$. Sorting of cells was performed on a MoFlo (Dako Cytomation, Carpinteria, CA, USA).

Immunoblotting, and cytospins

To visualize cells on cytospins May-Grunwald-Giemsa staining was used. Images were taken with an Olympus BX50 microscope (Olympus Nederland, Zoeterwoude, The Netherlands) using a 63x1.3 numeric aperture oil objective. For HIF2α Western blot analysis the primary antibody was from R and D Systems (R&D Systems Europe Ltd., Abingdon, United Kingdom) and it was used in 1:1000 dilutions. Secondary antibody was purchased from Dako Cytomation (Dako Cytomation, Glostrup, Denmark) and it was used in a 1:2000 dilution.

mRNA analysis

Using an RNeasy kit from Qiagen (Venlo, The Netherlands) total RNA was isolated according to the manufacturer's recommendations from sorted CB $CD34^+$ cells. For reverse transcription 200 ng of total RNA was used and cDNA was synthesized (Fermentas GmbH, St. Leon-Roth, Germany). Then cDNA was diluted 20-fold, and 4 µl was amplified using iQ SYBR Green Supermix (Bio-Rad, Veenendaal, The Netherlands) in a MyIQ thermocycler (Bio-Rad) and mRNA expression levels were quantified using MyIQ software (Bio-Rad). To normalize samples and calculate relative expression levels hypoxanthine phosphoribosyltransferase, RPL27 and RPL30 expression levels were used. Genome-wide expression analysis was performed on Illumina (Illumina, Inc., San Diego, CA) BeadChip arrays

(Sentrix Human-6; 46,000-probe sets). 1 μg of mRNA was combined from three independent transduction experiments and it was used for labeling reactions. Hybridization with the arrays was performed according to the manufacturer's instructions. Data were analyzed using the BeadStudio v3 gene expression module (Illumina, Inc.) and Genespring (Agilent, Amstelveen, The Netherlands).

Statistical analysis

All values are expressed as means ± SE. Student's *t* test was used for all other comparisons. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Overexpression of STAT5 imposes a long term proliferative advantage on HSCs but not on progenitors.

To gain further insight into whether STAT5-imposed long term self-renewal is exclusively restricted to the HSC population or whether long term self-renewal can also be imposed on progenitor cells we overexpressed a 4-hydroxytamoxifen (4-OHT)-inducible STAT5 retroviral vector in human cord blood (CB) $CD34^+$ cells.[32] CB $CD34^+$ cells were transduced with the MiNR1 empty vector or with the STAT5-wt ER retroviral vector after which cells were sorted into HSC and progenitor fractions using the following cell surface markers: $CD34^+/CD38^{low}$ (HSC), $CD38^+CD34^+CD45RA^-CD123^+$ (CMP), $CD38^+CD34^+CD45RA^+CD123^+$ (GMP) and $CD38^+CD34^+CD45RA^-CD123^-$ (MEP) (Figure 1A). The purity

of the sort was determined by CFC assays as reported earlier [33] and MEPs and GMPs were sorted to >95% homogeneity (data not shown). Q-PCR analysis was performed on myeloid and erythroid-specific genes and these studies also further confirmed good separation of HSC and MPP populations (Figure 1B). In particular CEBPα and PU.1 myeloid genes were detected in the CMP and GMP fraction while GATA-1 demonstrated a high expression in the MEP fraction. From each sorted cell population $4x10^4$ cells were plated in long-term MS5 stromal cocultures in the presence or absence of 4-OHT. Cultures were demi-depopulated weekly and expansion and differentiation of suspension cells were evaluated. As shown in Figure 1C, activation of STAT5 in HSCs resulted in a strong proliferative advantage over a period of 5 weeks. By the end of the fifth week cocultures initiated with STAT5-transduced HSCs displayed a 10 fold increase in cumulative expansion compared to MiNR1-transduced HSCs (Figure 1C). This increase in expansion was associated with the formation of cobblestone-area forming cells (CAFCs) underneath the MS5 stroma that appeared within 1 week after plating and continued to be present throughout the 5 week culture period (Figure 1D), and secondary cultures could be established from these week 5 CAFCs (data not shown).[34;35] In contrast, only a transient proliferative advantage was observed in the cocultures initiated with STAT5-transduced CMP and MEP populations and none of these progenitor cocultures were able to expand for longer than 3 weeks (Figure 1C). Interestingly, STAT5 overexpression did not induce increased expansion in GMPs (Figure 1C). In contrast to what was observed in HSCs transduced with STAT5, CAFCs were not observed in any of the progenitor subpopulations transduced with STAT5 (Figure 1D). The differentiation profile in the cultures was

determined from the suspension cells by MGG staining and FACS analysis. Overexpression of STAT5 in HSC, CMP and MEP compartments resulted in increased erythroid differentiation, whereby erythroblasts (in HSCs and CMPs) and orthochromatic normoblasts with distinct pyknotic nuclei (in MEPs) could be observed in MGG-stained cytospins at week 1 (Figure 1E). No erythroid differentiation was observed in STAT5-transduced GMPs (Figure 1E). FACS measurements were in agreement with these morphological data and indicated increased percentages of GPA-positive cells in the STAT5-transduced HSC, CMP and MEP fractions, while CD15 and CD11b percentages were decreased (Figure 1F). Expression of these cell surface markers was not changed significantly in cocultures initiated with GMPs transduced with STAT5 (Figure 1F). The presence of progenitors in the cocultures was evaluated weekly by performing CFC assays with suspension cells. At week 2 a strong increase in CFC frequency was observed in HSCs as well as CMPs and MEPs transduced with STAT5 (Figure 1G). At week 3, no CFCs were observed in any of the progenitor cultures, in line with the expansion data that indicated that after week 3 the cocultures initiated with transduced progenitor populations were exhausted (Figure 1G). Only cocultures initiated with HSCs were able to generate CFCs at week 3-5, whereby activation of STAT5 resulted in a strong increase in the number of CFCs (Figure 1F and data not shown).

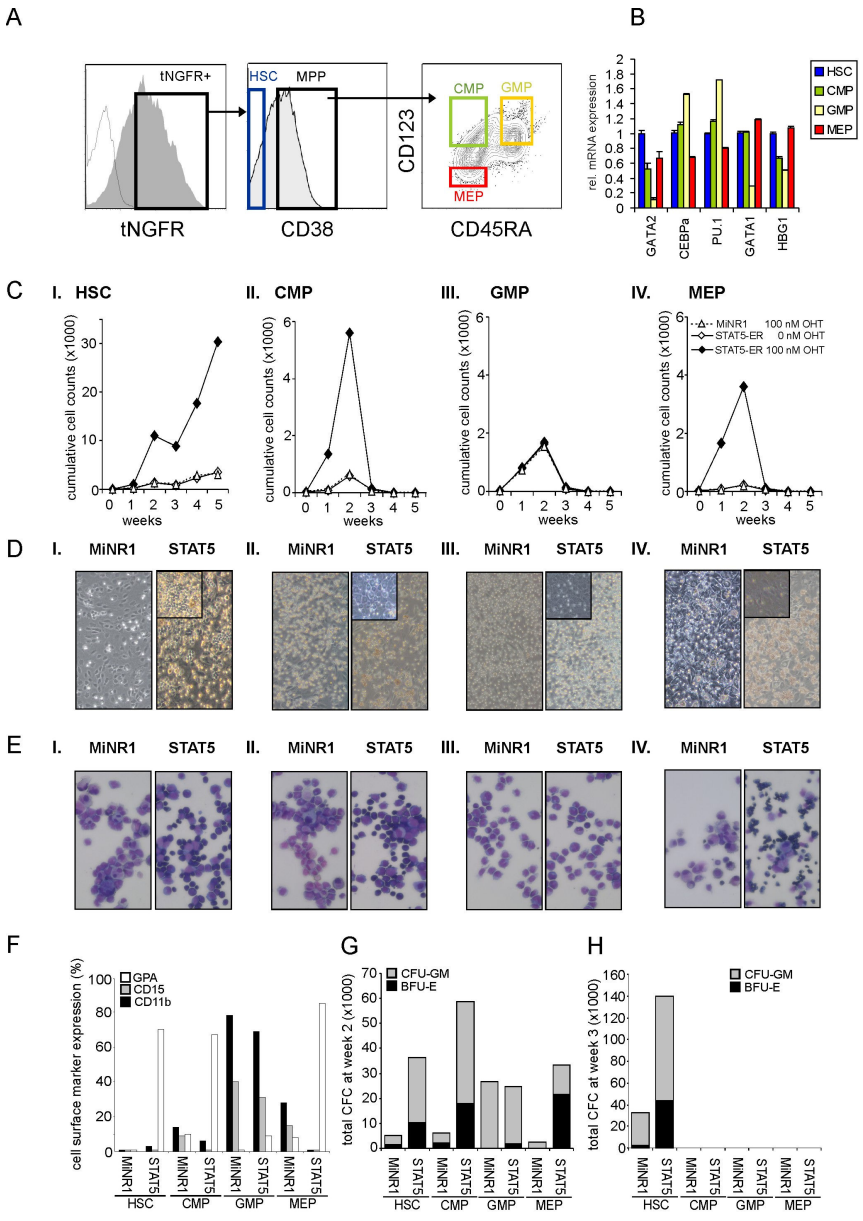

Figure 1. STAT5 imposes a long-term proliferative advantage on human HSCs but not progenitors in MS5 stromal cocultures. (A) Human CB CD34$^+$ cells were transduced with control and STAT5-wt ER retroviral vectors and tNGFR positive cells were sorted into HSC, common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP)

and megakaryocyte-erythroid progenitors (MEP) populations. (B) RNA was extracted from each sorted population and was used for qRT-PCR analysis. (C) Sorted HSCs (I), CMPs (II), GMPs (III) and MEPs (IV) were plated on MS5 stromal cells, stimulated with 4OHT as indicated and cultures were weekly demi-depopulated for analysis. Weekly cumulative cell counts are shown for representative experiment out of tree independent experiments. (D) Representative images from MS5 cocultures at week 2 as described in C. (E) MGG-stained cytospins from week one suspension cells from cocultures as described in C. (F) FACS analysis on suspension cells from MS5 cocultures as decribed in C at week 1. (G-H) Suspension cells from MS5 cocultures as described in C were analyzed for progenitor content by colony-forming cell (CFC) assay. Form each coculture at week two (G) and three (H) 2500 cells were plated in CFC assay in methylcellulose, colonies were evaluated two weeks after plating and total CFC numbers are shown.

Identification of STAT5 target genes in the HSC, CMP, GMP and MEP populations.

To identify STAT5 target genes in HSC and progenitor populations a genome-wide gene expression profiling was performed using Illumina BeadChip arrays. CB $CD34^+$ cells were transduced with the STAT5A-ER and MiNR1 control vectors and transduced cells were sorted into HSC and progenitor fractions. Sorted cells were stimulated with 100 nM 4-OHT for 24 hrs after which RNA was isolated. Three independent transductions and stem cell/progenitor sorts were performed and combined RNA fractions were used for hybridization with Illumina BeadChip arrays. Based on a gene expression change of >2-fold 187 STAT5 target genes were identified in the HSC compartment; 88 in the CMP fraction; 77 in the GMP fraction and 313 in the MEP fraction (Figure 2A and Supplemental Table 1). Remarkably, relatively little overlap existed between STAT5 target genes within the different stem cell and progenitor compartments, whereby the largest overlap in STAT5 target genes was observed between HSCs and MEPs

(23%, Figure 2B). These data suggest that STAT5 fulfils distinct functions within these different compartments of the hematopoietic system. A number of genes that were identified as STAT5 target genes within the HSC compartment were verified by Q-PCR analysis (Figure 2C). Next, we aimed to identify genes that would specifically associate with the long-term expansion and self-renewal phenotypes imposed on HSCs by STAT5. By downmodulating GATA1 in STAT5-transduced CB $CD34^+$ cells, we have been able to dissect the erythroid differentiation phenotypes from HSC self-renewal phenotypes[36] (Figure 2D). Lentiviral transduction of GATA1 RNAi vectors completely abrogated the STAT5-induced erythroid commitment, while long-term expansion, CAFC formation and HSC self-renewal remained intact. By using this approach we were able to identify 110 GATA1-independent STAT5 target genes.[37] By comparing this list of GATA1-independent target genes with the STAT5 target genes in HSCs we were able to identify 33 genes that would associate with long-term expansion and CAFC formation in the hematopoietic stem cell compartment (Figure 2E and Table 1). Data for HIF2α, GPR171, PIM1, OSM, TUBB1 and SOX21 is shown in figure 2F, and potential STAT5 binding sites within the promoters of these genes are indicated as well, whereby perfect palindromic STAT5 binding sites defined as $TTC(n_3)GAA$ are highlighted in green and imperfect palindromic STAT5 binding sites are not highlighted.

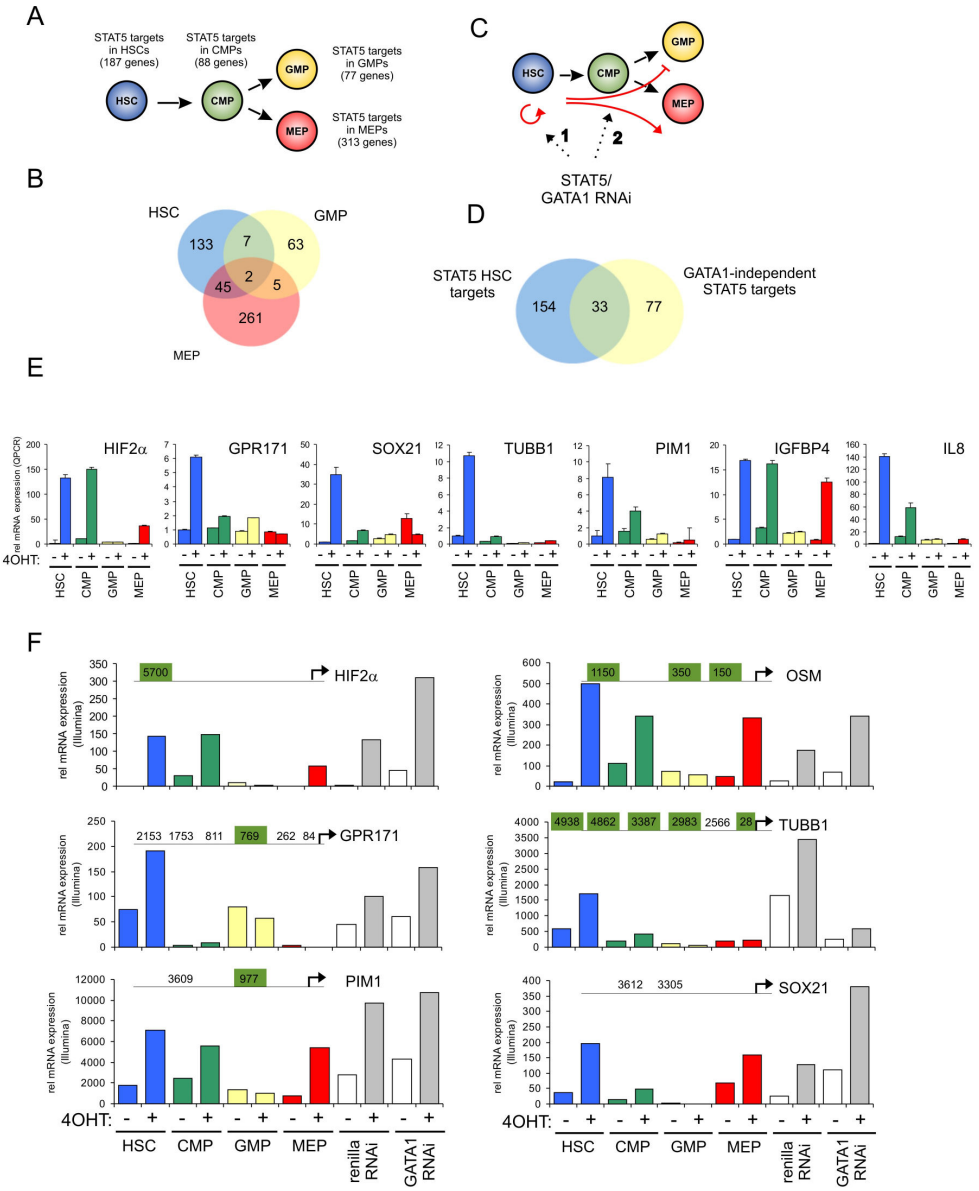

Figure 2. Identification of STAT5 target genes in HSCs and progenitors. CB cells were transduced with MiNR1 (control) and STAT5-wt ER retroviral vectors and sorted into HSC, CMP, GMP and MEP fractions. Cells were stimulated with 100 nM 4-OHT for 24 hours after which RNA was extracted which was used for Illumina BeadChip array analysis. Significantly expressed STAT5 target genes in HSC and progenitor compartments

(>2-fold change in gene expression) are shown. (B) Venn diagram shows STAT5 target genes in HSC, GMP and MEP fractions. (C) Verification of STAT5 target genes by qRT-PCR. (D) Schematic representation of the identification of GATA1-independent STAT5 target genes. CB $CD34^+$ cells were transduced with MiNR1 or STAT5A-ER and Scrambled RNAi or GATA1 RNAi vectors as described previously (Wierenga et al, Blood 2010). Cells were stimulated with 100 nM 4-OHT for 24 hours after which RNA was extracted which was used for Illumina microarray analysis. Thus, significantly expressed STAT5 target genes could be identified in the absence or presence of GATA1 (>2-fold change in gene expression) (E) Venn diagram in which STAT5 target genes in HSC and GATA1-independent target genes are visualized. (F) Dataset from the Illumina BeadChip arrays show expression of STAT5 target genes. Perfect palindromic STAT5 binding sites defined as TTC(n_3)GAA are highlighted in green and imperfect palindromic STAT5 binding sites are not highlighted.

Table 1. GATA1-independent STAT5 target genes in human CB $CD34^+$ HSCs

Symbol	Accession number	Fold change	Definition
C10orf128	XM_931112.2	2.56	chromosome 10 open reading frame 128
C14orf139	XR_017875.1	2.03	chromosome 14 open reading frame 139
CFH	NM_001014975.1	2.40	complement factor H (CFH), transcript variant 2
CISH	NM_145071.1	3.65	cytokine inducible SH2-containing protein
CXCR4	NM_003467.2	0.47	chemokine (C-X-C motif) receptor 4
EPAS1	NM_001430.3	176.75	endothelial PAS domain protein 1
ETV5	NM_004454.1	0.46	ets variant gene 5 (ets-related molecule)
FAM23B	NM_001013629.1	2.50	family with sequence similarity 23, member B
FCGR2B	XM_938851.1	2.07	Fc fragment of IgG, low affinity IIb, receptor
FILIP1L	NM_014890.2	3.40	filamin A interacting protein 1-like
FILIP1L	NM_182909.2	2.87	filamin A interacting protein 1-like
FRRS1	NM_001013660.2	2.51	ferric-chelate reductase 1
GJA4	NM_002060.2	0.47	gap junction protein, alpha 4
GPR171	NM_013308.3	2.52	G protein-coupled receptor 171
GYPA	NM_002099.3	2.32	glycophorin A
GYPB	NM_002100.3	3.70	glycophorin B
GYPE	NM_198682.2	3.80	glycophorin E
GYPE	NM_002102.3	3.62	glycophorin E
HBE1	NM_005330.3	4.69	hemoglobin, epsilon 1
HBZ	NM_005332.2	3.61	hemoglobin, zeta

Hs.551847	BE612775	2.00	cDNA clone IMAGE:3856355 5
HSPA6	NM_002155.3	3.48	heat shock 70kDa protein 6.
IL18RAP	NM_003853.2	2.76	interleukin 18 receptor accessory protein
LOC285016	NM_001002919.2	3.57	hypothetical protein LOC285016
MGLL	NM_007283.5	0.49	monoglyceride lipase
NRXN2	NM_138734.1	0.43	neurexin 2
OSBP2	NM_030758.3	3.17	oxysterol binding protein 2
OSM	NM_020530.3	22.21	oncostatin M
PIM1	NM_002648.2	4.05	pim-1 oncogene
RAP1GAP	NM_002885.1	4.06	RAP1 GTPase activating protein
SMAD6	NM_005585.3	0.48	SMAD family member 6
SOD2	NM_000636.2	2.19	superoxide dismutase 2
SOX21	NM_007084.2	5.11	SRY (sex determining region Y)-box 21
TMEM158	NM_015444.2	6.04	transmembrane protein 158
TUBB1	NM_030773.2	2.96	tubulin, beta 1

STAT5-induced long-term expansion, CFC numbers and LTC-IC frequencies are impaired by downmodulation of HIF2α.

One of the genes that was significantly up regulated by STAT5 in HSCs in a GATA1-independent manner was HIF2α, which was confirmed both at the mRNA level (Figure 2C and 3A) as well as at the protein level (Figure 3B). While HIF2α could also be upregulated by STAT5 in CMPs, only little upregulation was observed in MEPs and no expression was observed in GMPs (Figure 2C and 2F). In contrast no change in HIF1 mRNA expression was detected. At normoxia (21% O_2), very little HIF2α protein was observed in control cells (Figure 3B), possibly due to a combination of low levels of mRNA as well as degradation of hydroxylated HIF2α under these high oxygen conditions. In contrast, STAT5 activation was sufficient to induce robust protein expression of HIF2α at normoxia, which at least in part was mediated via increased mRNA levels, but might also involve stabilization of the HIF2α protein (Figure 3B). To further study the involvement of HIF2α in the STAT5-induced phenotypes we

downmodulated HIF2α using a lentiviral RNAi approach. CB $CD34^+$ cells were co-transduced with MiNR1 control or STAT5-ER vectors together with pLVUT scr-RNAi (scrambled control) or HIF2α-RNAi vectors. Double transduced cells were sorted and after 24 hrs of stimulation with 100 nM 4-OHT total RNA was isolated from each sorted population. Downmodulation of HIF2α was confirmed by Q-PCR (Figure 3A) as well as by Western blotting (Figure 3B). Next, long-term cocultures on MS5 bone marrow stromal cells were initiated using double transduced CB $CD34^+$ cells. Downmodulation of HIF2α in STAT5-transduced CB cells resulted in a remarkable decrease in expansion throughout the five week culture period (Figure 3C). As expected, HIF2α downmodulation in control CB $CD34^+$ cells did not significantly change the proliferation. Differentiation was also evaluated by FACS (CD11b, CD15, GPA, and CD71) and MGG staining of cytospins, but these experiments revealed that downmodulaton of HIF2α did not change the differentiation pattern in either the STAT5-transduced or in the MiNR1 control cells (data not shown). The decrease in expansion of STAT5-transduced cells upon downmodulation of HIF2α was also reflected by reduced CFC numbers at week one, three and five (Figure 3D). There was no difference observed in the distribution of colony-forming unit granulocyte-macrophage (CFU-GM) or burst-forming unit erythroid (BFU-E) colonies. To determine whether decreased expansion was related to an increase in apoptosis Annexin-V staining was performed, but no significant increase in Annexin-V positive cells was observed upon downmodulation of HIF2α in any of the groups (data not shown). Finally, stem cell frequencies were determined in long-term culture-initiating cell (LTC-IC) assays under limiting dilution conditions. Cells expressing activated STAT5 had an

approximately four-fold increase in LTC-IC frequency compared to controls (n=3; MiNR1/Scr RNAi: 1/1322±99; STAT5/Scr RNAi: 1/341±43, Figure 3E). When HIF2α was downmodulated in STAT5-transduced cells, LTC-IC frequencies were decreased approximately two to three-fold compared to STAT5 transduced cells (n=3; STAT5/Scr RNAi: 1/341±43, STAT5/HIF2αRNAi: 1/836±63, Figure 3E).

A number of HIF target genes have been described that might potentially mediate the HIF2α-induced phenotypes, including VEGF, cMYC and p21. We could indeed confirm that VEGF and cMYC expression was downmodulated upon transduction with HIF2α-RNAi vectors, both in MiNR1 control as well as in STAT5-transduced CB $CD34^+$ cells (Figure 3F). In contrast, while p21 was upregulated by STAT5 as observed previously, cotransduction with HIF2α-RNAi vectors rather resulted in a further increase in expression of p21 (Figure 3F). No effects of STAT5 or HIF2a-RNAi were observed on the expression of HIF1α, HIF1β, or VHF (data not shown).

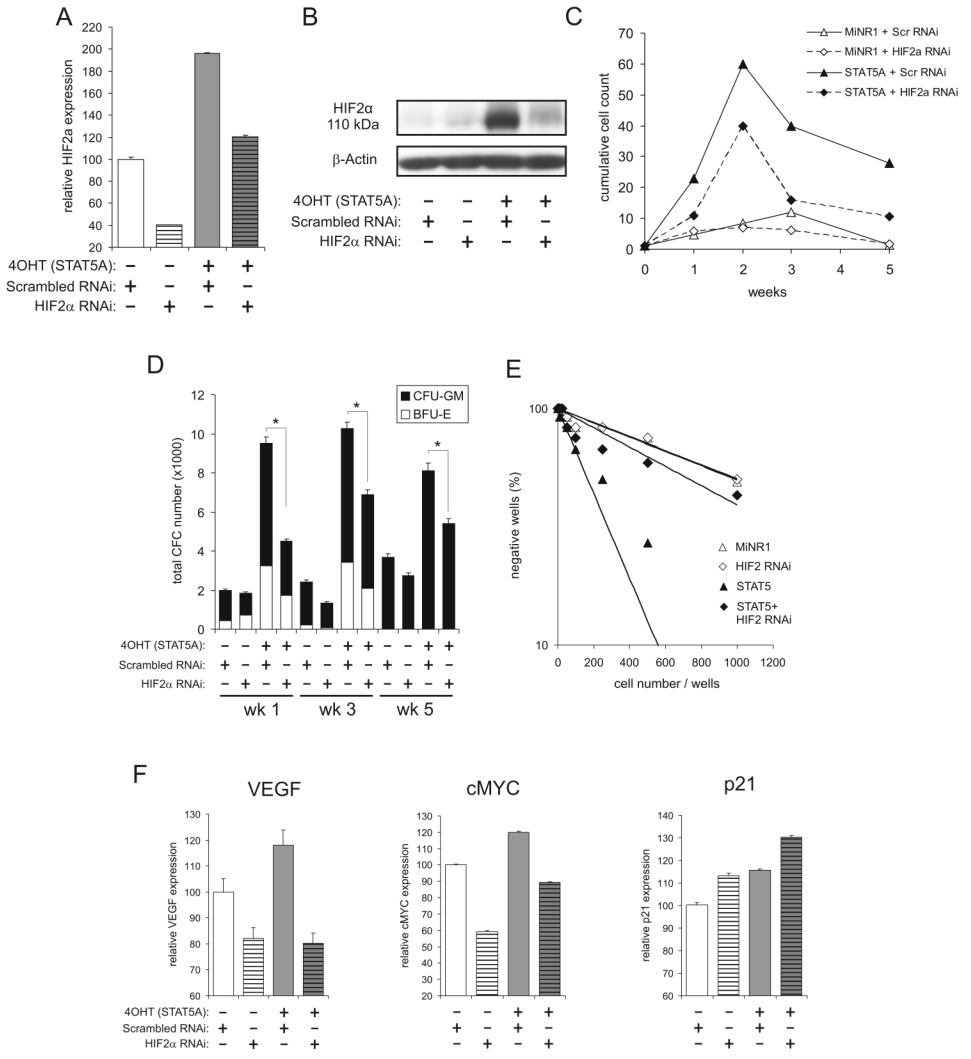

Figure 3. STAT5-induced long-term proliferation, increased CFC and LTC-IC frequencies is impaired by downmodulation of HIF2α. (A) CB CD34$^+$ cells were transduced with MiNR1 control or STAT5-wt ER together with scrambled RNAi control or HIF2αRNAi constructs and double transduced cells were sorted. Sorted cells were stimulated with 100 nM 4-OH for 24 hr after which RNA was isolated. HIF2α mRNA expression levels were measured by Q-PCR. (B) CB CD 34$^+$ cells ells were transduced and sorted as described in (A) but were now used for Western blot analysis to determine HIF2α protein levels. (C) Double transduced CB CD34$^+$ cells as described in A were cultured in

MS5 coculture. Cocultures were demi-depopulated weekly and cumulative cell counts are indicated. (D) Suspension cells from cocultures as described in C were analyzed in CFC assays. Data indicates total CFC numbers from a representative experiment out of three independent experiments. (E) LTC-IC frequencies were determined in limiting dilution on MS5 stromal cells. Cultures were cultured for five weeks after which methylcellulose was added. At week 7 LTC-IC frequencies were determined. Data shows the stem cell frequencies of a representative experiment out of three independent experiments. (F) RNA was isolated from double transduced cells as described in A, RNA was isolated and qRT-PCR was performed to identify HIF2α target genes.

Discussion

While various studies have highlighted the important role that STAT5 fulfils in both normal hematopoiesis as well as in the development of leukemia, remarkably little has been revealed regarding the potential molecular mechanisms that are involved. Our data now show that the STAT5-imposed long-term proliferative advantage and cobblestone formation is restricted to the HSC compartment. STAT5 was unable to induce long-term growth and cobblestone formation in myeloid or erythroid progenitor cells. Progenitor-initiated cultures showed only a transient STAT5-induced increase in cell numbers and they could not be maintained longer than 3 weeks regardless of STAT5 activity. To identify genes involved in the STAT5-induced phenotypes we performed a genome-wide gene expression profiling on stem and progenitor cells which resulted in the identification of 33 STAT5 target genes in the HSC compartment. One of significantly altered genes was HIF2α. When HIF2α was downmodulated in STAT5 activated cells we observed that expansion of STAT5/HIF2α RNAi-transduced cells on MS5

bone marrow stromal cocultures was reduced, coinciding with reduced CFC and LTC-IC frequencies, while differentiation was not affected.

Recently, we demonstrated that the STAT5-induced long-term self-renewal phenotypes could be dissected from the STAT5-induced erythropoiesis by lentiviral downmodulation of GATA1.[38] This allowed the identification of STAT5-induced target genes associated with erythroid commitment as well as target genes that did not associate with erythropoiesis. By comparing this last gene set with the list of target genes that were induced by STAT5 in HSCs, we were able to identify 33 genes that might potentially play a role in the long-term self-renewal phenotype induced by STAT5. HIF2α was studied further in detail because it has been shown to be overexpressed in various malignancies, it plays a role in malignant transformation and it was overexpressed with the highest fold increase in the HSC compartment. Our data indicated that downregulation of HIF2α reduced STAT5-induced cell proliferation, CFC numbers and LTC-IC frequencies, while apoptosis or differentiation was not affected Thus, the long-term phenotypes induced by STAT5 in HSCs are, at least in part, mediated via HIF2α. So far HIF2 was especially linked to be expressed in endothelial cells but the present study demonstrates that it can also be expressed HSC in the setting of constitutive activation of STAT5. Under normoxic conditions, proline residues of Hypoxia-Induced Factor 2 are hydroxylated resulting in a reduction in protein levels via VHL-mediated proteasomal degradation. Under hypoxic conditions, such as in the presumed endosteal quiescent stem cell niche, HIFs are stabilized and act as transcription factors.[39] It is currently unknown whether HIF-induced target genes are essential to maintain stemness of HSCs, but it has become clear that HIF transcription factors are upregulated

in a variety of tumors.[40-43] While HIF2α is normally only stabilized under hypoxic conditions, robust levels of HIF2α protein were observed in STAT5-transduced HSCs, even under normoxia. This was mediated via an upregulation of HIF2α mRNA levels, but might also involve stabilisation of the protein at normoxia, for instance via upregulation of reactive oxygen species (ROS),[44] as we have indeed observed increased ROS levels in STAT5-transduced cells (unpublished observations). HIF2α target genes might include those that provide adaptation to hypoxic stress and facilitate a transition from oxidative phosphorylation to glycolysis as a mode of ATP production. Amongst others, HIF2α activates genes involved in glucose uptake such as Glut1, as well as cMYC, OCT4, p21, and VEGF.[45;46] Although not completely understood, enhanced glycolysis has been observed in many tumors, even under conditions when there is enough O_2 available, and it is conceivable that HIF2α contributes to proliferation of STAT5-transduced HSCs via similar mechanisms as well. In human CB $CD34^+$ cells, we also find that cMYC and VEGF are upregulated in STAT5-transduced cells in a HIF2α-dependent manner, and although so far PIM1 has only been described as a HIF1α target gene[47;48], we do find that PIM1 is upregulated in STAT5-transduced HSCs as well. Besides the growth-promoting activity of these genes, it has been suggested that HIF2α might contribute to growth of tumor cells via activation of EGFR and IGFR1 tyrosine kinases.[49] Future studies will reveal the role of these HIF2α-induced target genes and signal transduction pathways in the long-term growth and self-renewal phenotypes that STAT5 imposes on HSCs, and it will be interesting to elucidate whether targeting of HIF2α will be sufficient to impair self-renewal of STAT5-transfomed leukemic stem cells as well.

The phenotype of the leukemic stem cell is under continuous debate, and also it is not always clear whether leukemias arise from genetic mutations in HSCs or in progenitors. In mice, a fatal MPD could be induced when activating STAT5 mutants were expressed in the primitive $CD34^{-}LSK$ population, but not when expressed in committed progenitors.[50] Apparently, STAT5 is not sufficient to re-install a self-renewal program on progenitor cells that have already lost their self-renewal potential. This is in contrast to other oncogenes such as MLL-AF9 or MLL-ENL, which, particularly when expressed at high levels [51] can efficiently transform progenitor cells by re-activating an "embryonic stem cell-like" gene expression program.[52-54] Amongst others, this gene expression program includes Myb, Hmgb3 and Cbx5, which next to the HoxA/Meis gene expression program appear to be sufficient to impose self-renewal on progenitor cells and ultimately induce leukemic transformation. We indeed did not observe an upregulation of these genes in STAT5-transduced HSCs or MPPs. In line with our observations, it has become clear that the BCR-ABL fusion protein also lacks the capability to reprogram more differentiated progenitor sells.[55] More recently, using transgenic models in which BCR-ABL p210 was exclusively expressed in the immature $Sca1^{+}$ compartment, it was shown that expression only in HSCs but not progenitors is sufficient to induce leukemic transformation.[56]

In our microarray analyses we observed that STAT5 target genes did not overlap significantly amongst HSCs, CMPs, GMPs and MEPs. Thus, our data show that STAT5 activates quite different sets of genes in HSC and progenitors which could explain why the STAT5 induced phenotypes are distinct in the different compartments. In our coculture assays we observed that no growth advantage was imposed on GMPs. This is somewhat

remarkable since various myeloid growth factors such as IL3 and GM-CSF are quite efficient in activating STAT5 in myeloid cells. Initially, in STAT5 $^{-/-}$ animals no defects were observed in myelopoiesis, suggesting that STAT5 is dispensable for myeloid development [57], although later it was shown that granulopoiesis under myelosuppressive conditions was impaired.[58] Further studies are required to elucidate why constitutive activation of STAT5 appears insufficient to alter phenotypes in GMPs under steady-state conditions. Furthermore, it is noteworthy that while STAT5 activation efficiently induced erythroid commitment from HSC, CMP and MEP compartments, in line with previously published data [59-61], no erythroid differentiation could be induced by STAT5 in GMPs. Apparently, GMPs have differentiated beyond a stage at which an erythroid-associated gene expression program can be induced by STAT5. Although it still needs to be elucidated why STAT5 target genes within HSC, CMP, GMP and MEP compartments are so diverse, it is plausible that a tissue-specific expression of STAT5 cofactors, repressors, additional transcription regulators or the epigenetic status of gene regulatory elements are involved, which will certainly be a focus in future studies.

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Chapter 4

Downregulation of signal transducer and activator of transcription 5 (STAT5) in CD34$^+$ cells promotes megakaryocytic development, whereas activation of STAT5 drives erythropoiesis

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Summary

Although it has been proposed that the common myeloid progenitor gives rise to granulocyte/monocyte progenitors and megakaryocyte/erythroid progenitors (MEP), little is known about molecular switches that determine whether MEPs develop into either erythrocytes or megakaryocytes. We used the thrombopoietin receptor c-Mpl, as well as the megakaryocytic marker CD41, to optimize progenitor sorting procedures to further subfractionate the MEP ($CD34^+CD110^+CD45RA^-$) into erythroid progenitors ($CD34^+CD110^+CD45RA^-CD41^-$) and megakaryocytic progenitors ($CD34^+CD110^+CD45RA^-CD41^+$) from peripheral blood. We have identified signal transducer and activator of transcription 5 (STAT5) as a critical denominator that determined lineage commitment between erythroid and megakaryocytic cell fates. Depletion of STAT5 from $CD34^+$ cells by a lentiviral RNAi approach in the presence of thrombopoietin and stem cell factor resulted in an increase in megakaryocytic progenitors (CFU-Mk), whereas erythroid progenitors (BFU-E) were decreased. Furthermore, an increase in cells expressing megakaryocytic markers CD41 and CD42b was observed in STAT5 RNAi cells, as was an increase in the percentage of polyploid cells. Reversely, overexpression of activated STAT5A(1*6) mutants severely impaired megakaryocyte development and induced a robust erythroid differentiation. Microarray and quantitative reverse transcription-polymerase chain reaction analysis revealed changes in expression of a number of genes, including GATA1, which was downmodulated by STAT5 RNAi and upregulated by activated STAT5.

Introduction

In the human system, megakaryocytes and erythrocytes arise from a common megakaryocyte/erythroid progenitor (MEP) that is derived from a common myeloid progenitor (CMP) [1] or possibly emerge via a more direct pathway from hematopoietic stem cells (HSCs).[2] The induction of MEP to the erythroid or megakaryocytic lineage is regulated by extrinsic factors, such as erythropoietin (EPO) and thrombopoietin (TPO), and by an intrinsic program of transcription factors.[3;4] Two major transcription factors have been identified that are involved in CMP differentiation, namely, GATA-1, which drives differentiation of MEP, and PU.1, which regulates granulocyte-monocyte precursors (reviewed in [1]). The downregulation of PU.1 expression in the CMP is the first event associated with the restriction of differentiation to erythroid and megakaryocytic (MK) lineages.[3] Instruction of the MEP toward the erythroid or MK lineage is dependent on the gene dosage of GATA-1.[5] Loss of GATA-1 leads to differentiation arrest and apoptosis of erythroid progenitors and accumulation of immature megakaryocytes.[6-8] Similarly, low expression of GATA-1 in mice favors the megakaryocytic development [9], whereas forced GATA-1 expression reprogrammed the common lymphoid and myeloid progenitors to the MK/erythroid lineage.[10] These instructive programs of GATA-1 are executed in association with a number of additional cofactors that reside in close proximity to GATA sequences in MK-specific promoters, such as the Ets family factors, or act independently, such as the proto-oncogene c-myb. [4;5] The identification of JAK-2 mutation in patients with polycythemia vera (PV) and essential thrombocythemia (ET) [11-15] has focused attention on the

involvement of signal transducer and activator of transcription 5 (STAT5) in erythroid and megakaryocytic development.[16] Because of the constitutive activation of JAK2, a number of downstream targets are persistently activated, including STAT5 and ERK.[16] So far, STAT5 has been identified as a relevant transcription factor for the erythroid and stem cell compartment.[17-19] Forced overexpression of STAT5 in $CD34^+$ cord blood (CB) cells results in an expansion of the erythroid lineage, inhibits myeloid differentiation in part because of downregulation of C/EBP-α, and results in a two- to threefold expansion of the stem cell compartment.[20;21] Knockdown studies using STAT5 RNAi constructs in CB $CD34^+$ cells have shown that a reduced STAT5 expression impairs the number of stem and progenitor cells, as determined by long-term culture-initiating cell and colony-forming cell (CFC) assays, without affecting the differentiation program.[18]

A number of recent studies have demonstrated that the commitment to the erythroid or MK lineage is dependent on the gene dose of relevant transcription factors. This not only applies to GATA-1 but also has been demonstrated for the Ets family of transcription factors and Runx1. Therefore, in the present study, we tested whether the gene dosage of STAT5 is relevant for MK or erythroid development by performing STAT5 RNAi and STAT5A(1*6) overexpression studies in $CD34^+$ cells that had been cultured with TPO and stem cell factor (SCF). The results demonstrate that downregulation of STAT5 impairs erythroid differentiation and promotes MK development.

Materials and methods

Cell Culture and Purification of $CD34^+$ Cells

Mo7e cells were routinely propagated in RPMI 1640 (BioWhittaker, Lonza, Verviers, Belgium, http://www.lonza.com) supplemented with heat-inactivated fetal calf serum (FCS) (5%, vol/vol; Sigma-Aldrich, Zwijndrecht, The Netherlands, http://www.sigmaaldrich.com) and interleukin (IL)-3 (10 ng/ml). CD34-positive cells were obtained from healthy donors undergoing granulocyte colony-stimulating factor (G-CSF) treatment following institutional guidelines. $CD34^+$ cells were isolated by EasySep immunomagnetic cell selection procedures (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) as described by the manufacturer. To generate MK cells, $CD34^+$ cells were grown in HPGM (Cambrex, Walkersville, MD, http://www.cambrex.com) supplemented with 40 ng/ml TPO (a kind gift from Kirin Brewery Co. [Tokyo, http://www.kirin.co.jp/english]) and 40 ng/ml SCF (Immunex, Seattle, http://immunex.com). To induce erythroid differentiation, $CD34^+$ cells were grown in HPGM medium supplemented with 1 U/ml EPO and 40 ng/ml SCF. Cells were counted by trypan blue exclusion using a hemocytometer twice weekly, and fresh medium plus cytokines was added.

Flow Cytometry Analysis and Sorting Procedures

Sorting of the $CD34^+$ cells into progenitor fractions was performed on the basis of the combinatorial expression of cell surface antigens, as previously reported [22;23]: CMP as $CD34^+CD110^-CD45RA^-$, granulocyte/monocyte progenitors (GMP) as $CD34^+CD110^-CD45RA^+$, and MEP as

$CD34^+CD110^+CD45RA^-$. In addition, the MEP was separated in two fractions on the basis of the $CD41^+$ expression. Erythroid progenitors consisted of $CD34^+CD110^+CD45RA^-CD41^-$ cells, whereas megakaryocyte progenitors consisted of $CD34^+CD110^+CD45RA^-CD41^+$ cells. The fluorescence-activated cell sorting analyses were performed on a FACSCalibur (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com), and sorting of the cells was performed on a MoFlo (DakoCytomation, Carpinteria, CA, http://www.dakocytomation.com). Antibodies were obtained from Becton Dickinson, except phycoerythrin-labeled anti-CD41a and anti-CD42b, which were purchased from CLB (Amsterdam, The Netherlands, http://www.sanquin.nl). The CD61-positive cell fraction was purified from primary cultures after 7 days by MoFlo sorting as indicated. Data were analyzed using WinList 3D (Verity Software House, Topsham, ME, http://www.vsh.com) and FlowJo (Tree Star, Ashland, OR, http://www.treestar.com) software.

CFC Progenitor Assays

CFC assays were performed in 1.2% methylcellulose containing 30% FCS, 57.2 μM β-mercaptoethanol, and 2 mM glutamine, supplemented with 20 ng/ml IL-3, 20 ng/ml IL-6, 20 ng/ml G-CSF, 20 ng/ml c-Kit ligand, and 1 U/ml EPO (Eprex-Cilag, Brussels, Belgium, http://www.cilag.ch/indexE.htm). CFU-GM and BFU-E assays were performed in 1.2% methylcellulose containing 30% FCS, 57.2 μM β-mercaptoethanol, and 2 mM glutamine, supplemented with 10 ng/ml granulocyte-macrophage colony-stimulating factor and 10 ng/ml IL-3 or 2 U/ml EPO, respectively.

A commercially available kit for evaluation of colony-forming units-megakaryocytic progenitors (CFU-Mk) was used according to the manufacturer's instructions (MegaCult-C medium with cytokines; StemCell Technologies). In short, 2,000 purified CD34-positive cells were seeded per double chamber culture slide in serum-free medium containing thrombopoietin (50 ng/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml), and collagen (1.1 mg/ml). Cultures were incubated for 10-12 days, followed by dehydration and immunocytochemical staining of the slides. Megakaryocyte colonies were detected using the CD41 and alkaline phosphatase detection system and counterstained in Evan's Blue. Cultures were scored for the presence of pure MK colonies consisting of at least five nucleated cells expressing CD41, as previously described.[24] For polyploidization analysis, cells were analyzed on cytospins, and multinucleated cells were counted.

Retroviral Transductions

293T human embryonic kidney cells (2.5 x 10^6 cells) were transfected with 3 μg of pCMV Δ8.91, 0.7 μg of VSV-G, and 3 μg of pTRIP Renilla RNAi or pTRIP STAT5 RNAi (kind gifts of Dr. H. Spits, Department of Cell Biology and Histology, Amsterdam Medical Center, Division of Immunology, Netherlands Cancer Institute, Amsterdam, The Netherlands). Target sequences have been described by Scheeren et al. [25]. Twenty-four hours after transient transfection, medium was changed to HPGM, and after 12 hours, supernatant containing lentiviral particles was harvested and stored at -80°C. Mo7e cells were cultured in RPMI 1640 supplemented with 10% FCS and 10 ng/ml IL-3 for 4 hours at 37°C and 5% CO_2, and $CD34^+$ cells were cultured in HPGM supplemented with c-Kit ligand, Flt-3 ligand

(both from Amgen, Thousand Oaks, CA, http://www.amgen.com), and TPO (100 ng/ml each) for 16 hours at 37°C and 5% CO_2 prior to transductions. Transductions of $CD34^+$ cells were performed in two consecutive rounds of 8-12 hours with lentiviral supernatant supplemented with c-Kit ligand/Flt-3 ligand/TPO (100 ng/ml each) and polybrene (4 μg/ml). Mo7e cells were transduced in one round of 12 hours with lentiviral supernatant supplemented with 10% FCS, 10 ng/ml IL-3, and 4 μg/ml polybrene. Transduction efficiency was measured by fluorescence-activated cell sorting (FACS) analysis, and knockdown was investigated by means of Western blot. Alternatively, knockdown was investigated by means of quantitative reverse transcription (RT)-polymerase chain reaction (PCR). Retroviral transductions with constitutively activated STAT5A(1*6) were performed as described previously.[20]

Preparation of Cell Lysates and Western Blotting

Total cell lysates were performed by centrifugation of equal amounts of cells followed by lysing cell pellets in Laemmli sample buffer. SDS-polyacrylamide gel electrophoresis and immunoblotting were performed according to standard procedures. Antibodies used for Western blotting were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, http://www.scbt.com; antibodies against STAT5, ERK, and β-Actin). Antibodies against tyrosine-phosphorylated STAT5 (pY694, C11C5) were obtained from Cell Signaling Technology (Leiden, The Netherlands, http://www.cellsignal.com). Detection was performed according to the manufacturer's guidelines (ECL; Amersham Biosciences, Little Chalfont, U.K., http://www.amersham.com).

Quantitative PCR and Illumina Microarray Analysis

Target gene expression was investigated by means of quantitative reverse transcription-polymerase chain reaction (Q-PCR). Total RNA was isolated from 1 x 10^5 to 1 x 10^6 cells using the RNeasy kit from Qiagen (Venlo, The Netherlands, http://www1.qiagen.com) according to the manufacturer's recommendations. RNA was reverse transcribed with M-MuLV reverse transcriptase (Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com). For real-time RT-PCR, 2-µl aliquots of cDNA were real-time amplified using iQ SYBR Green Supermix (Bio-Rad, Veenendaal, The Netherlands, http://www.bio-rad.com) on a MyIQ thermocycler (Bio-Rad) and quantified using MyIQ software. As a negative control, RNA minus reverse transcriptase prepared cDNA was used in PCRs. To verify the correct sizes of PCR fragments, 10-µl aliquots were run on 1.5% agarose gels. β-Actin expression was used to normalize expression of all genes investigated. Genome-wide expression analysis was performed on Sentrix Human-6 BeadChip Arrays (Illumina, Inc., San Diego, http://www.illumina.com) (46,000 probe sets) on an outsource basis by ServiceXS (Leiden, The Netherlands, http://www.servicexs.com). mRNA (0.5-1 mg) was used in labeling reactions and hybridization with the arrays according to the manufacturer's instructions. Data were analyzed using the BeadStudio v3 Gene Expression Module (Illumina).

Statistical Analysis

Data were expressed as mean ± SEM. Differences between samples were calculated using Student's *t* test. A two-sided *p* value of <.05 was considered statistically significant.

RESULTS

STAT5 Downregulation Promotes Megakaryocytic Expansion from CD34$^+$ Cells

To determine the efficacy of STAT5 downmodulation by our lentiviral RNAi approach, the Mo7e cell line was transduced using Renilla RNAi control vectors or STAT5 RNAi vectors that target both STAT5A and STAT5B.[18;24] Cell lysates were prepared for Western blot analysis 2 days after the transduction procedure, and as depicted in Figure 1A, 70%-80% downregulation of STAT5 protein could be obtained. As loading control, blots were stripped and reprobed against ERK1 and ERK2, and no differences in expression were observed (Fig. 1A). Similarly, normal CD34$^+$ cells isolated from peripheral blood were transduced with the STAT5 or Renilla RNAi constructs, sorted, and cultured with TPO and SCF (Fig. 1B). Q-PCR analysis of STAT5 mRNA after 10 days of culture demonstrated a 60%-70% reduction in STAT5 expression (Fig. 1B). To study the cellular consequences of the reduced STAT5 expression, transduced CD34$^+$ cells (n = 3) were cultured in CFU-MK, CFU-GM, and erythroid progenitors (BFU-E) assays. Downregulation of STAT5 resulted in a 1.9-fold increase in the number of megakaryocytic progenitors (CFU-MKs) compared with CD34$^+$ cells transduced with the control vector (Fig. 1C; p = .002). No change was observed in the number of CFU-GM, whereas a 0.6-fold reduction in BFU-E colony formation was shown (Fig. 1C; p < .01). Furthermore, megakaryocytic differentiation was monitored in serum-free liquid culture assays driven by SCF and TPO. Under these conditions, a fivefold expansion was reached within 2 weeks (Fig. 1D), and 20%-50% of the cells

expressed the megakaryocyte markers CD41 and CD42b (Fig. 1E). Although the expansion was unaffected by STAT5 downmodulation (Fig. 1D), an increase in cells expressing CD41 (42% ± 12% vs. 52% ± 15%; p = .002) and CD42b (19% ± 10% vs. 30% ± 15%; p = .002) was observed under these conditions (numbers indicate the average of the individual data sets shown in Fig. 1E). In addition, STAT5 downregulation resulted in an approximately threefold increase of polyploid cells (Fig. 1F, 1G). Together, these data indicate that downmodulation of STAT5 impairs the development of erythroid progenitors and promotes megakaryocytic differentiation.

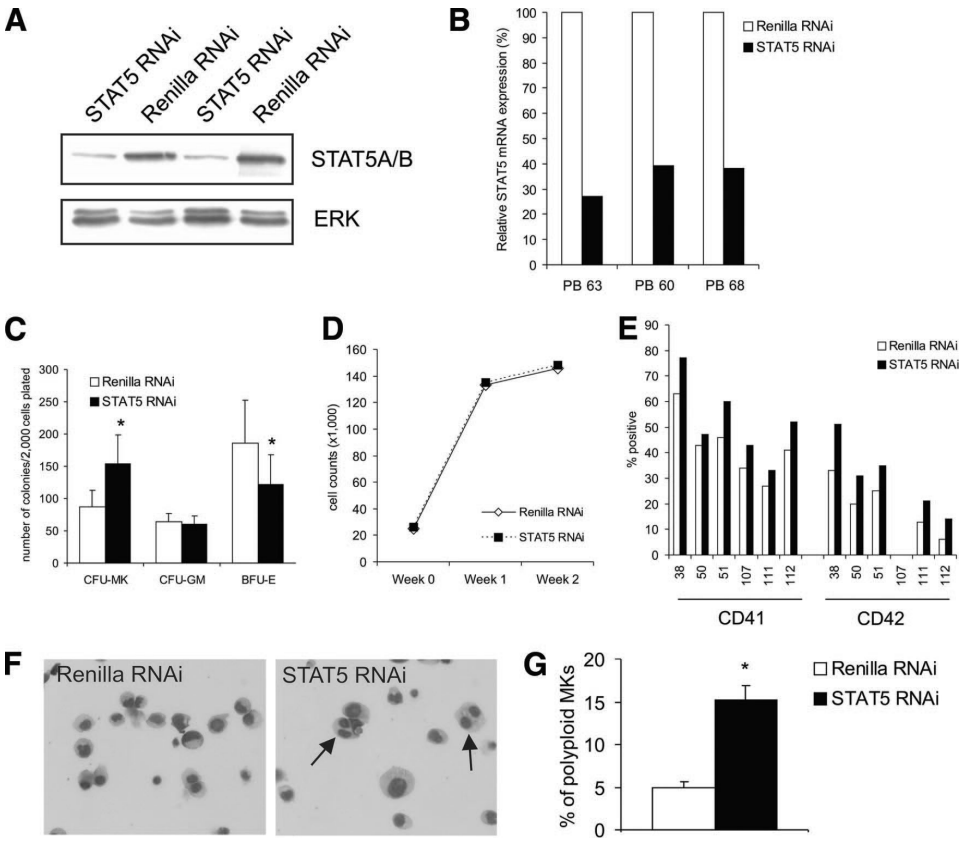

Figure 1. STAT5 downmodulation promotes MK differentiation of peripheral blood (PB) CD34$^+$ cells. (A): Mo7e cells were stably transduced with STAT5 or Renilla lentiviral RNAi vectors, and downmodulation of STAT5 expression was determined by Western blotting. Blots were stripped and reprobed for ERK to determine equal loading. Data from two individually transduced cell lines are shown. (B): PB CD34$^+$ cells were transduced with STAT5 or Renilla lentiviral RNAi vectors, and transduced cells were MoFlo-sorted on the basis of green fluorescent protein (GFP) expression, after which RNA was isolated and the levels of STAT5 expression were determined in three independent transduction experiments by quantitative reverse transcription-polymerase chain reaction (PB 60, 63, and 68). (C): Transduced PB CD34$^+$ cells were plated in progenitor assays as described in Materials and Methods (*, $p < .05$). (D): Transduced cells were plated in serum-free HPGM supplemented with 40 ng/ml thrombopoietin and stem cell factor, and expansion was

monitored weekly. (E): Megakaryocyte differentiation of transduced cells expanded as in (D) was monitored by fluorescence-activated cell sorting for the expression of CD41 and CD42b. Numbers indicated at the *x*-axis represent individual PB $CD34^+$ cells that were expanded. Percentages are within the GFP^+-transduced populations. (F): Representative images of cytospins from expanded cells as in (D). (G): The percentage of polyploidy cells was determined by counting polyploidy cells from 30 images taken randomly from cytospins (*, $p < .05$). Abbreviations: MK, megakaryocytic; STAT, signal transducer and activator of transcription.

Constitutive Active STAT5A(1*6) Induces Erythroid Differentiation at the Expense of Megakaryocyte Development

As the studies described above indicated that reduction of STAT5 is sufficient to promote megakaryopoiesis and impair erythropoiesis, we wished to study the reverse process by elevating STAT5 activity. $CD34^+$ cells were transduced with constitutively active STAT5A(1*6), and MoFlo-sorted green fluorescent protein-positive cells were cultured in suspension with TPO and SCF. As depicted in Figure 2A, STAT5A(1*6) overexpression induced a strong proliferative advantage over MiGR1 control cells. Whereas megakaryocytic differentiation was induced in control cells, a strong reduction in CD41- and CD42b-positive cells was observed in the STAT5A(1*6)-transduced group (Fig. 2B). Instead, erythroid differentiation was induced by active STAT5, as demonstrated by the strong increase in CD36 and GPA-positive cells (Fig. 2B). Representative FACS profiles are shown in Figure 2C, and the induction of erythropoiesis was confirmed by morphological analysis of week 2 cells (Fig. 2D). Thus, these data indicate that constitutive active STAT5A(1*6) induces erythroid differentiation at the expense of megakaryocyte development.

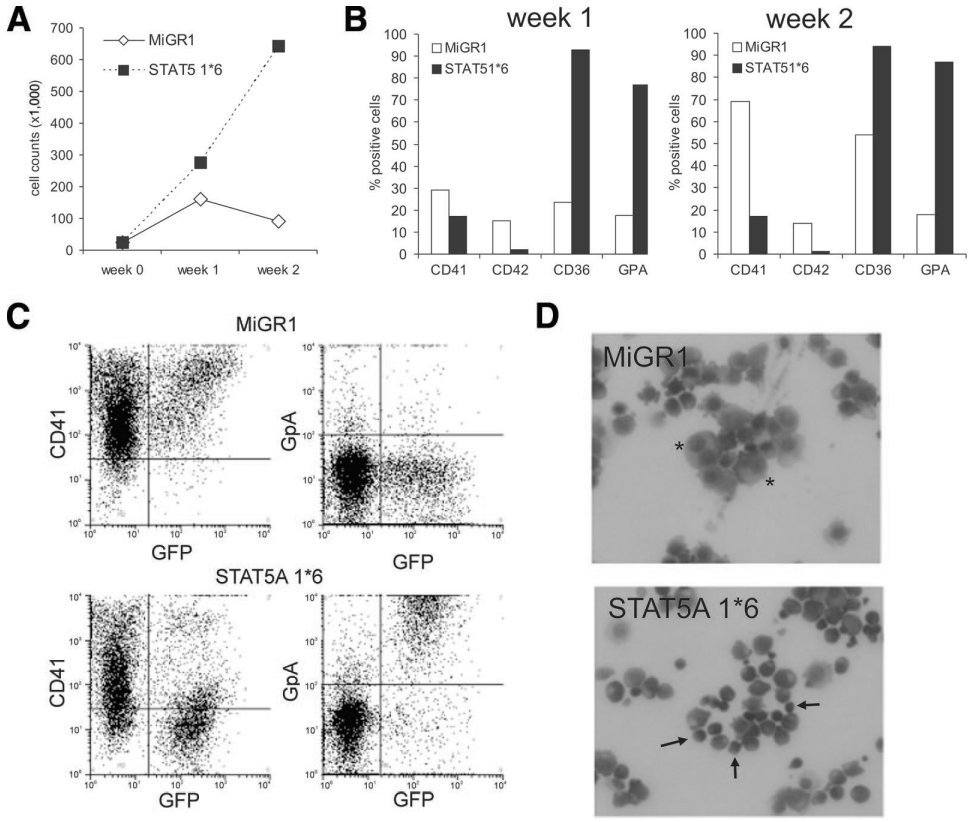

Figure 2. Activation of STAT5 impairs megakaryopoiesis and induces erythropoiesis. (A): Peripheral blood $CD34^{+}$ cells were transduced with STAT5A(1*6) or empty control MiGR1 vectors, and cells were expanded in serum-free HPGM supplemented with 40 ng/ml stem cell factor and thrombopoietin. Expansion was monitored weekly. (B): Expansion assays were performed as in (A), and differentiation was monitored by fluorescence-activated cell sorting (FACS) at weeks 1 and 2. (C): Representative FACS profiles at week 2. Percentages are within the GFP+-transduced populations. (D): Representative images of cytospins at week 2. Asterisks indicate polyploid cells, and arrows indicate erythroid normoblasts. Abbreviations: GFP, green fluorescent protein; STAT, signal transducer and activator of transcription.

Involvement of Endogenous STAT5 in TPO-Induced Megakaryopoiesis and EPO-Induced Erythropoiesis

Megakaryopoiesis was induced by stimulating $CD34^+$ cells with TPO and SCF. Differentiation was monitored by FACS analysis, and as shown in Figure 3A, a strong increase in $CD41^+$ cells was observed, whereas no increase in GPA^+ cells was detected. STAT5 expression levels were determined throughout the differentiation period, and no changes in expression levels were observed (Fig. 3B). Some low levels of STAT5 tyrosine phosphorylation were observed in the initial phase of the experiment that could no longer be observed after 7 days of differentiation (Fig. 3B). In response to EPO, GPA^+ cells were efficiently generated, and the percentage of $CD41^+$ cells was reduced (Fig. 3C). Furthermore, a strong upregulation of STAT5 expression was observed in response to EPO, which coincided with a strong upregulation of STAT5 tyrosine phosphorylation (Fig. 3D). These data indicate that the endogenous STAT5 expression and activation patterns coincide with erythroid commitment in response to EPO.

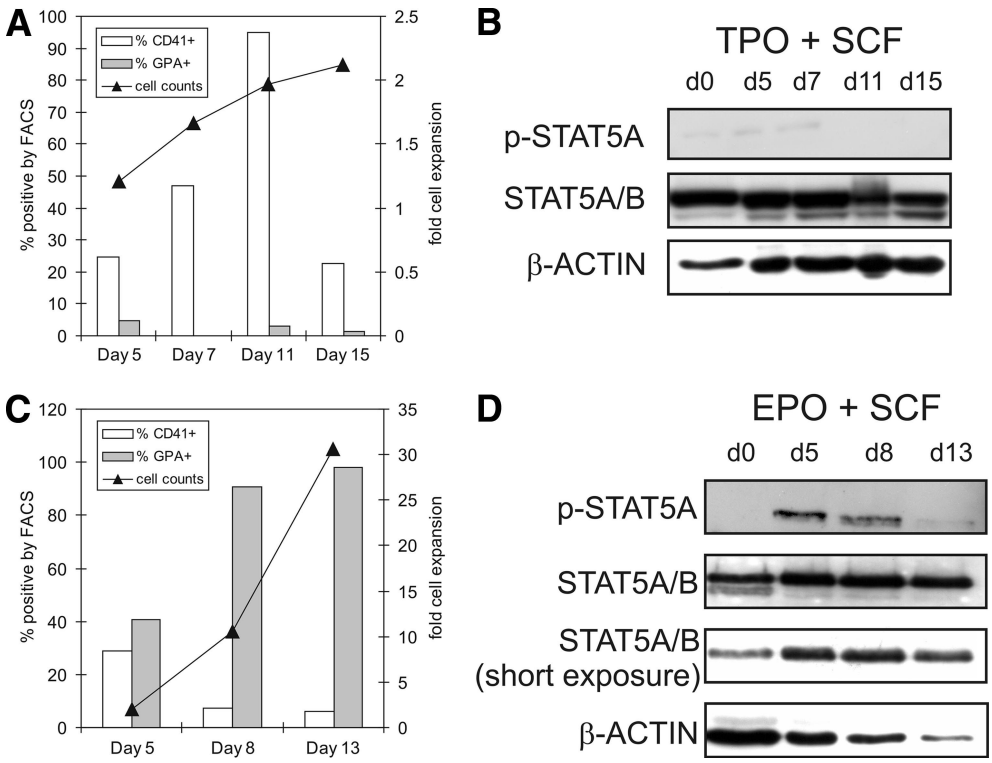

Figure 3. Involvement of endogenous STAT5 in TPO-induced megakaryopoiesis and EPO-induced erythropoiesis. (A): Peripheral blood $CD34^+$ cells were grown in HPGM supplemented with TPO and SCF (40 ng/ml each), and differentiation was monitored by FACS for CD41 and GPA. Relative expansion is also shown on the right axis, and cell numbers at the start were set to 1. (B): Experiment was performed as in (A), but now aliquots were taken from the cultures at the indicated time points, and STAT5 expression and tyrosine phosphorylation were determined by Western blotting. As loading control, blots were probed with antibodies against β-Actin. (C): Experiment was performed as in (A), but now cells were stimulated with 1 U/ml EPO and 40 ng/ml SCF. (D): Western blotting experiments were performed as in (B), but now cells were stimulated with 1 U/ml EPO and 40 ng/ml SCF. Abbreviations: d, day; EPO, erythropoietin; FACS, fluorescence-activated cell sorting; SCF, stem cell factor; STAT, signal transducer and activator of transcription; TPO, thrombopoietin.

Optimalization of Megakaryocyte Progenitor (CFU-MK) Sorting Protocols

To establish protocols to isolate megakaryocyte progenitors, $CD34^+$ cells were first isolated from the peripheral blood of healthy donors, after which CMP, GMP, and MEP were sorted on the basis of CD45RA and CD110 expression, as shown in Figure 4A. Progenitor assays were performed with sorted cells, and as shown in Figure 4B and 4C, these sorting procedures yielded a high purity of myeloid and erythroid populations. The $CD34^+/CD110^+/CD45RA^-$ MEP population was further sorted into $CD41^{high}$ and $CD41^{low}$ populations (Fig. 4A). As shown in Figure 4B and 4C, the $CD34^+/CD110^+/CD45RA^-/CD41^+$ population contained the majority of CFU-MK progenitors (73%), whereas the majority of the BFU-E was retained in the $CD34^+/CD110^+/CD45RA^-/CD41^-$ population (85%). Thus, we propose that the MEP compartment can be further subdivided into erythroid and megakaryocytic progenitors on the basis of CD41 expression (schematically depicted in Fig. 4D). The progenitor frequencies that we observed ranged from 5% to 10%, approximately, indicating that within the defined FACS gates, we also isolated cells that were most likely differentiated beyond the progenitor stage.

To further study the specificity of our sorting procedures, Mk progenitors were sorted and CFU-MK assays were initiated with TPO in the absence or presence of EPO. Megakaryocytic progenitors were generated by TPO, and the number of CFU-MKs was not affected by addition of EPO (Fig. 4E). Sorted Mk progenitors were also differentiated in liquid culture conditions, and upon stimulation with TPO and SCF, multinucleated megakaryocytes were readily generated (Fig. 4F). In contrast, no megakaryocytes could be

generated from sorted Mk progenitors in response to EPO and SCF, and cells retained an immature megakaryocytic-erythroid progenitor morphology (Fig. 4F). Reversely, erythroid progenitors were sorted and CFC assays were performed in the absence or presence of EPO, and these experiments revealed that BFU-E were generated in an EPO-dependent manner (Fig. 4G). In liquid culture conditions, erythroid progenitors could be differentiated toward an erythroid fate in response to EPO, whereas an immature progenitor phenotype was retained in the presence of TPO (Fig. 4H). Thus, these data indicate that the $CD34^{+}/CD110^{+}/CD45RA^{-}/CD41^{+}$ Mk population responds to TPO and can be differentiated toward mature megakaryocytes, whereas the $CD34^{+}/CD110^{+}/CD45RA^{-}/CD41^{-}$ erythroid progenitor (Ery) population predominantly responds to EPO

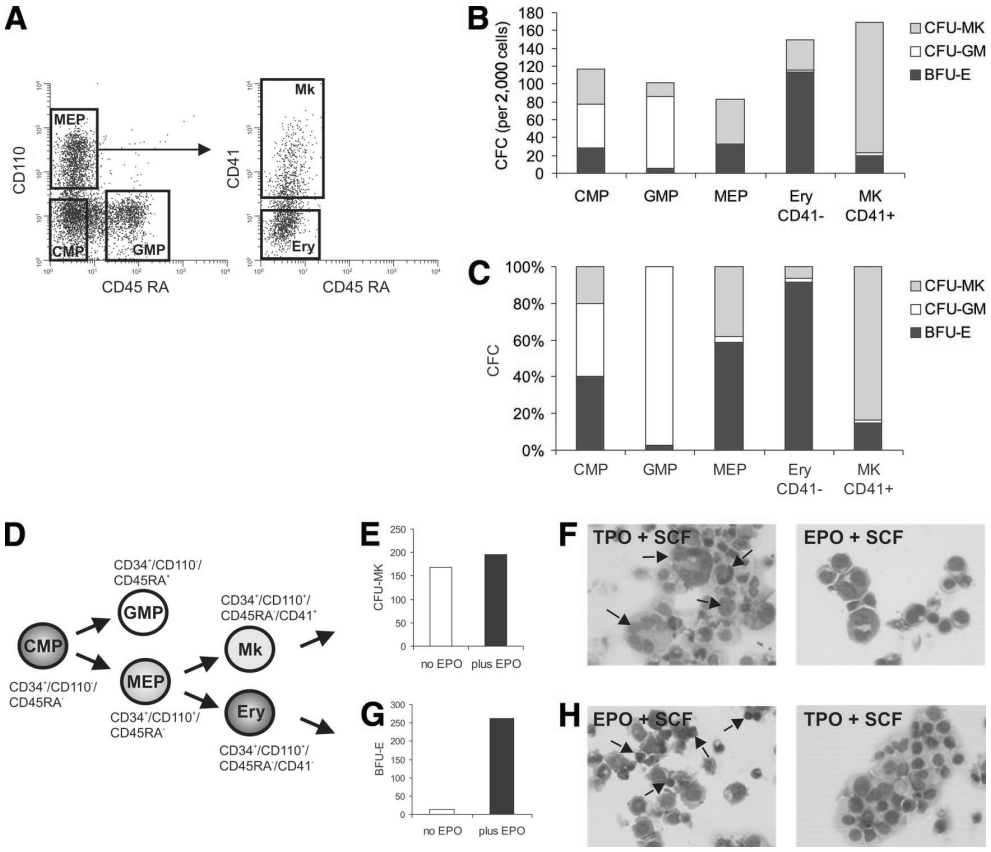

Figure 4. Mk sorting procedures. (A): Peripheral blood $CD34^+$ cells were isolated by EasySep immunomagnetic cell selection followed by MoFlo sorting on the basis of CD45RA, CD110, and CD41 expression. (B): Sorted populations were plated in progenitor assays as described in Materials and Methods. (B) indicates the total numbers of progenitors per 2,000 plated cells within the various sorted compartments as indicated. In (C), data are represented in percentages. (D): Overview of marker expression on various progenitor subsets: CMP ($CD34^+CD110^-CD45RA^-$), GMP ($CD34^+CD110^-CD45RA^+$), MEP ($CD34^+CD110^+CD45RA^-$), Ery ($CD34^+CD110^+CD45RA^-CD41^-$), and Mk ($CD34^+CD110^+CD45RA^-CD41^+$). (E): $CD41^+$ MK progenitors were sorted as indicated in (A), and CFU-MK assays were performed in the absence or presence of EPO. (F): Experiment was performed as in (E), but now $CD41^+$ MK progenitors were sorted and cultured in liquid culture conditions supplemented with TPO/SCF or EPO/SCF as

indicated. Differentiation was monitored by MGG staining of cytospins at day 10. (G): Erythroid $CD41^-$ progenitors were sorted as indicated in (A), and BFU-E assays were performed in the presence or absence of EPO. (H): Experiment was performed as in (G), but now $CD41^-$ Erys were sorted and cultured in liquid culture conditions supplemented with EPO/SCF or TPO/SCF as indicated. Differentiation was monitored by MGG staining of cytospins at day 10. Abbreviations: CMP, common myeloid progenitor; EPO, erythropoietin; Ery, erythroid progenitor; GMP, granulocyte/monocyte progenitor; MEP, megakaryocyte/erythroid progenitor; Mk, megakaryocytic progenitor; MK, megakaryocytic; SCF, stem cell factor; TPO, thrombopoietin.

STAT5 Downregulation in CMP, GMP, MEP, MK, and Erythroid Progenitors

Since the effects of STAT5 downregulation were observed in the balance between the megakaryocytic and erythroid lineage, we questioned whether we could further identify these modulating effects by isolation of megakaryocytic and erythroid progenitors. Therefore, $CD34^+$ cells were transduced with STAT5 RNAi or Renilla RNAi control vectors and then further sorted in CMP ($CD110^-/CD45RA^-$), GMP ($CD110^-/CD45RA^+$), MEP ($CD110^+/CD45RA^-$), MK ($CD110^+/CD45RA^-/CD41^+$), and erythroid ($CD110^+/CD45RA^-/CD41^-$) progenitors. Subsequently, the effects of STAT5 downregulation were studied on the progenitor frequency and composition of the different subsets. As presented in Figure 5A and 5B in two representative transductions with $CD34^+$ cells, a clear reduction in BFU-E and an increase in CFU-MKs was observed upon downmodulation of STAT5, whereas the number of myeloid progenitors was much less affected.

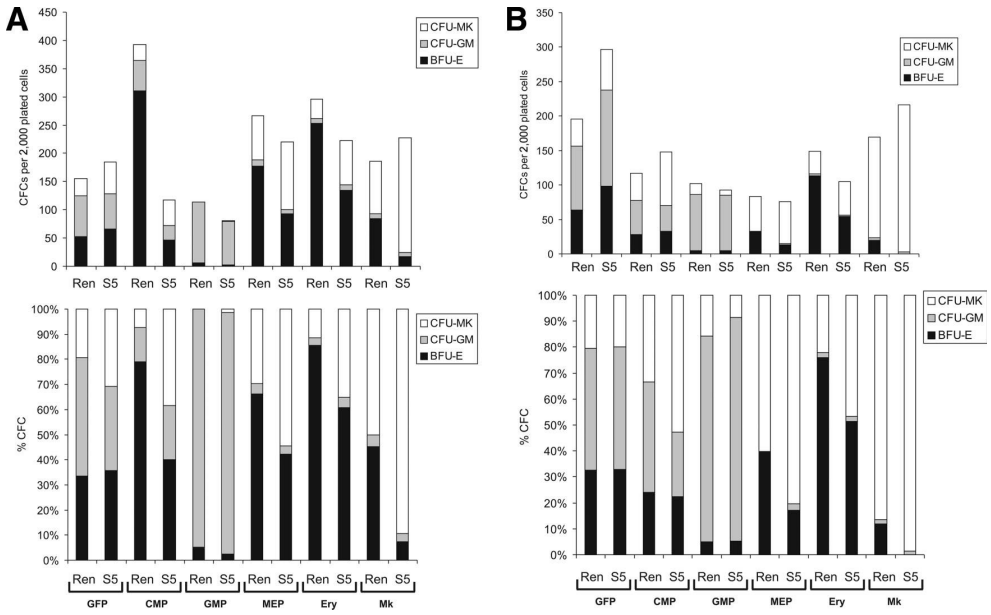

Figure 5. Signal transduced and activator of transcription 5 (STAT5) downmodulation induces a shift from MEP to Mk progenitors.

Peripheral blood $CD34^+$ cells were prestimulated for 16 hours and transduced with STAT5 or Ren RNAi lentiviral vectors in two rounds followed by sorting into CMP, GMP, MEP, Ery, and Mk progenitor subsets as well as GFP expression on day 4. Progenitor assays were performed as described in Materials and Methods. Cells were plated into methylcellulose directly after sorting. Two independent transductions and progenitor assays are shown in (A) and (B). Upper panels represent number of CFCs per 2,000 plated cells; lower panels represent the data as percentages. Abbreviations: CFC, colony-forming cell; CMP, common myeloid progenitor; Ery, erythroid progenitor; GFP, green fluorescent protein; GMP, granulocyte/monocyte progenitor; MEP, megakaryocyte/erythroid progenitor; Mk, megakaryocytic progenitor; Ren, Renilla; S5, signal transducer and activator of transcription 5.

Microarray Analysis on Transduced PB CD34$^+$ Cells

To correlate the effects of STAT5 depletion on megakaryoctye/erythroid lineage fate decisions with changes in gene expression, we performed microarray analysis on STAT5- and Renilla RNAi-transduced CD34$^+$ cells that were expanded under serum-free conditions with SCF and TPO. Upon downmodulation of STAT5 expression, 53 genes were downregulated and 33 genes were upregulated (Table 1; fold change, >2; $p < .05$). The list of downmodulated genes contained a large number of genes involved in regulating erythropoiesis, including GATA1; hemoglobin ε1, γG, γA, and β1; CD36; and glycophorin B. Furthermore, known STAT5 target genes, such as Pim2, were found to be downregulated in STAT5 RNAi cells. Q-PCR analysis confirmed that Gata1 expression is reduced upon downmodulation of STAT5, and the same was true for the STAT5 target gene Bcl-XL and the cell cycle inhibitor p21 (Fig. 6A). Importantly, many of these genes were found to be significantly upregulated in cells expressing constitutively activated STAT5A(1*6), including GATA1 and p21 (Fig. 6B). Moreover, in line with the erythroid commitment induced by STAT5, we observed a strong upregulation of hemoglobins (Fig. 6B). The microarray analysis further revealed reduced expression of CXCR4 and the tetraspanin family members CDC151 and TSPAN4 upon downmodulation of STAT5, and inhibition of these membrane proteins has been associated with enhanced megakaryocyte development.

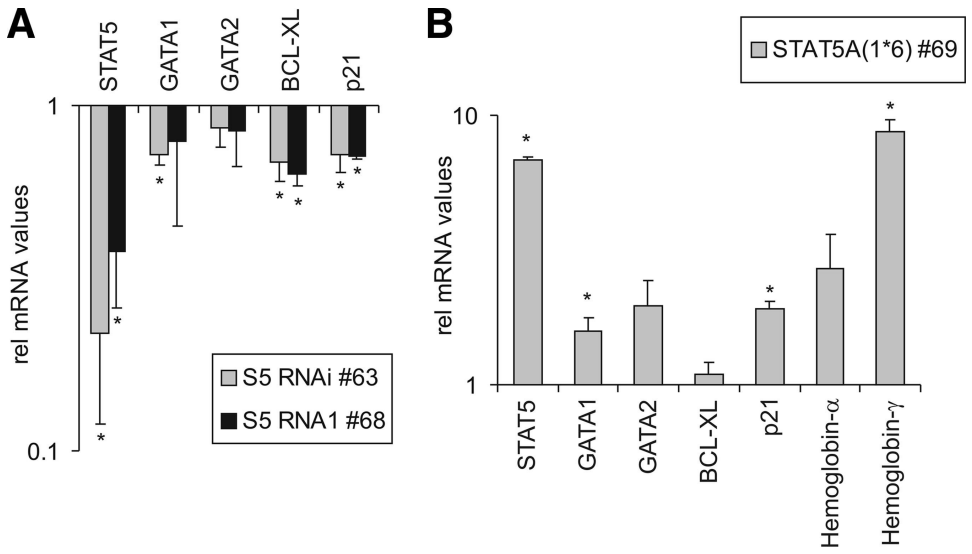

Figure 6. Quantitative reverse transcription-polymerase chain reaction (Q-PCR) analysis on transduced peripheral blood (PB) CD34$^+$ cells.

(A): PB CD34$^+$ cells were transduced with STAT5 or Renilla RNAi lentiviral vectors (PB 63 and 68), and RNA was isolated for Q-PCR analysis of the indicated target genes. Data represent fold change in target gene expression of STAT5 RNAi relative to Renilla RNAi control transduced cells (*, $p < .05$). (B): PB 69 was transduced with STAT5A(1*6) or MiGR1 control vectors, and RNA was isolated for Q-PCR analysis of the indicated target genes. Data represent fold change in target gene expression of STAT5A(1*6) relative to MiGR1 control transduced cells (*, $p < .05$). Abbreviations: rel, relative; S5, STAT5, signal transducer and activator of transcription 5.

Table 1. Downregulated and upregulated genes in STAT5 RNAi-transduced PB CD34$^+$ cells

Accession no.	Gene symbol	Gene name	[S5]/[ren]	Significance
NM_199242.1	UNC13D	*unc-13 homolog D*	0.33	Exocytosis
NM_005330.3	HBE1	*Hemoglobin, ε 1*	0.36	Erythropoiesis
NM_182687.1	PKMYT1	*Protein kinase, membrane associated tyrosine/threonine 1*	0.38	Regulation of cyclin dependent protein kinase activity
NM_017723.1	FLJ20245	*Hypothetical protein FLJ20245*	0.39	Defense response to pathogen
NM_000184.2	HBG2	*Hemoglobin, γ G*	0.40	Erythropoiesis
NM_152236.1	GAS2L1	*Growth arrest-specific 2 like 1*	0.40	Cell cycle arrest
NM_003748.2	ALDH4A1	*Aldehyde dehydrogenase 4 family, member A1*	0.40	Proline biosynthesis
NM_013241.1	FHOD1	*Formin homology 2 domain containing 1*	0.41	Cell organization and biogenesis
NM_031471.4	URP2	*UNC-112 related protein 2*	0.41	Cell adhesion
NM_138768.2	MYEOV	*Myeloma overexpressed gene*	0.41	
NM_003152.2	STAT5A	*Signal transducer and activator of transcription 5A*	0.42	HSC self-renewal, erythropoiesis
NM_000559.2	HBG1	*Hemoglobin, γ A*	0.42	Erythropoiesis
NM_199002.1	ARHGEF1	*Rho guanine nucleotide exchange factor (GEF) 1*	0.42	Rho protein signal transduction, proliferation
NM_006238.2	PPARD	*Peroxisome proliferative activated receptor, Δ*	0.42	Cell proliferation, fatty acid catabolism
NM_006875.2	PIM2	*pim-2 oncogene*	0.42	Cell proliferation
NM_198252.1	GSN	*Gelsolin (amyloidosis, Finnish type)*	0.43	Actin filament polymerization
NM_153246.2	MGC45491	*Hypothetical protein MGC45491*	0.43	
NM_001001548.1	CD36	*CD36 antigen*	0.44	Erythropoiesis
NM_015518.1	ULK3	*unc-51-like kinase 3*	0.44	Protein serine/threonine kinase activity
NM_002213.3	ITGB5	*Integrin, β 5*	0.44	Cell-matrix adhesion
NM_002049.2	GATA1	*GATA binding protein 1 (globin transcription factor 1)*	0.44	Erythropoiesis
NM_015927.3	TGFB1I1	*Transforming growth factor β 1 induced transcript 1*	0.44	Positive regulation of transcription
NM_015481.1	ZNF385	*Zinc finger protein 385*	0.44	Regulation of transcription
NM_005762.2	TRIM28	*Tripartite motif-containing 28*	0.44	Protein ubiquitination
NM_002928.2	RGS16	*Regulator of G-protein signalling 16*	0.44	Regulation of G-protein coupled receptor protein signaling
NM_002100.3	GYPB	*Glycophorin B (MNS blood group)*	0.45	Erythropoiesis
NM_003775.1	EDG6	*Endothelial differentiation, G-protein-coupled receptor 6*	0.45	Immune response
NM_012342.2	BAMBI	*BMP and activin membrane-bound inhibitor homolog*	0.46	
NR_001589.1	HBBP1	*Hemoglobin, β pseudogene 1*	0.46	Erythropoiesis
NM_025106.2	SPSB1	*splA/ryanodine receptor domain and SOCS box containing 1*	0.46	Intracellular signaling cascade
NM_004945.2	DNM2	*Dynamin 2*	0.46	Endocytosis
NM_000118.1	ENG	*Endoglin (Osler-Rendu-Weber syndrome 1)*	0.46	Organ morphogenesis
NM_173587.2	RCOR2	*REST corepressor 2*	0.46	Regulation of transcription
NM_014216.3	ITPK1	*Inositol 1,3,4-triphosphate 5/6 kinase*	0.46	Signal transduction
NM_015270.2	ADCY6	*Adenylate cyclase 6*	0.46	cAMP biosynthesis
NM_032885.4	ATG4D	*ATG4 autophagy related 4 homolog D*	0.47	Autophagic vacuole formation
NM_015125.2	CIC	*Capicua homolog*	0.47	Regulation of transcription, DNA-dependent
NM_004419.3	DUSP5	*Dual specificity phosphatase 5*	0.47	Protein amino acid dephosphorylation
NM_002105.2	H2AFX	*H2A histone family, member X*	0.48	Chromosome organization and biogenesis
NM_016363.3	GP6	*Glycoprotein VI*	0.48	Platelet activation
NM_000203.2	IDUA	*Iduronidase, α-L*	0.48	Glycosaminoglycan metabolism
NM_032809.2	FAM73B	*Family with sequence similarity 73, member B*	0.48	
NM_153359.1	MGC24975	*Hypothetical protein MGC24975*	0.48	
NM_016215.3	EGFL7	*EGF-like-domain, multiple 7*	0.48	Regulation of cell migration
NM_001003693.1	C6orf21	*Chromosome 6 open reading frame 21*	0.48	
NM_020831.3	MKL1	*Megakaryoblastic leukemia (translocation) 1*	0.49	Regulation of transcription, DNA-dependent
NM_013355.3	PKN3	*Protein kinase N3*	0.49	Protein amino acid phosphorylation
NM_022749.4	RAI16	*Retinoic acid induced 16*	0.49	
NM_144671.2	FAM109A	*Family with sequence similarity 109*	0.49	
NM_004260.1	RECQL4	*RecQ protein-like 4*	0.49	Development, DNA repair
NM_006665.2	HPSE	*Heparanase*	0.49	Proteoglycan metabolism, inflammatory response
NM_030662.2	MAP2K2	*Mitogen-activated protein kinase kinase 2*	0.49	Protein serine/threonine kinase activity
NM_004188.2	GFI1B	*Growth factor independent 1B*	0.49	Cell proliferation, negative regulation of transcription
NM_015092.3	SMG1	*PI-3-kinase-related kinase SMG-1*	2.01	Phosphoinositide phosphorylation

(Continued)

Table 1. (Continued)

Accession no.	Gene symbol	Gene name	[S5]/[ren]	Significance
NM_153464.1	ILF3	*Interleukin enhancer binding factor 3*	2.02	Positive regulation of transcription, DNA-dependent
NM_032525.1	TUBB6	*Tubulin, β 6*	2.02	Microtubule-based movement
NM_006676.4	USP20	*Ubiquitin specific peptidase 20*	2.02	Ubiquitin-dependent protein catabolism
NM_006827.4	TMED10	*Transmembrane emp24-like trafficking protein 10*	2.07	Vesicle targeting, intracellular protein transport, ER to Golgi transport
NM_033656.2	BRWD1	*Bromodomain and WD repeat domain containing 1*	2.09	
NM_015642.2	ZBTB20	*Zinc finger and BTB domain containing 20*	2.09	Regulation of transcription, DNA-dependent
NM_000046.2	ARSB	*Arylsulfatase B*	2.09	Glycosaminoglycan metabolism
NM_032886.1	MGC15912	*Hypothetical protein MGC15912*	2.09	
NM_030798.2	WBSCR16	*Williams-Beuren syndrome chromosome region 16*	2.12	Ran guanyl-nucleotide exchange factor activity
NM_021230.1	MLL3	*Myeloid/lymphoid or mixed-lineage leukemia 3*	2.18	Regulation of transcription, DNA-dependent
NM_004906.3	WTAP	*Wilms' tumor 1 associated protein*	2.19	Nuclear membrane
NM_002231.3	CD82	*CD82 antigen*	2.21	Protein binding
NM_153001.1	PSMC4	*Proteasome (prosome, macropain) 26S subunit, ATPase, 4*	2.27	Proteolysis and peptidolysis
NM_004516.2	ILF3	*Interleukin enhancer binding factor 3*	2.30	Positive regulation of transcription, DNA-dependent
NM_012154.2	EIF2C2	*Eukaryotic translation initiation factor 2C, 2*	2.30	Protein biosynthesis
NM_194247.1	HNRPA3	*Heterogeneous nuclear ribonucleoprotein A3*	2.31	Nucleotide binding
NM_015339.2	ADNP	*Activity-dependent neuroprotector*	2.34	Regulation of transcription, DNA-dependent
NM_001029835.1	CCM2	*Cerebral cavernous malformation 2*	2.43	
NM_012469.2	C20orf14	*Chromosome 20 open reading frame 14*	2.44	RNA splicing
NM_001025234.1	TSPAN4	*Tetraspanin 4*	2.45	Protein complex assembly
NM_006821.3	ACOT2	*Acyl-CoA thioesterase 2*	2.47	Acyl-CoA metabolism
NM_006386.3	DDX17	*DEAD (Asp-Glu-Ala-Asp) box polypeptide 17*	2.74	RNA processing
NM_014411.2	NAG8	*Nasopharyngeal carcinoma associated gene protein-8*	2.75	
NM_020791.1	TAOK1	*TAO kinase 1*	2.80	Protein serine/threonine kinase activity
NM_002629.2	PGAM1	*Phosphoglycerate mutase 1*	3.03	Glycolysis
NM_178044.1	GIYD2	*GIY-YIG domain containing 2*	3.22	DNA repair
NM_005782.2	THOC4	*THO complex 4*	3.28	Nuclear mRNA splicing, mRNA nuclear export
NM_003671.2	CDC14B	*CDC14 cell division cycle 14 homolog B*	3.56	Protein amino acid dephosphorylation
NM_003467.2	CXCR4	*Chemokine (C-X-C motif) receptor 4*	4.00	HSC homing, G-protein coupled receptor protein signaling pathway
NM_001013251.1	SLC3A2	*Solute carrier family 3, member 2*	4.03	Calcium ion transport, cell growth
NM_004357.3	CD151	*CD151 antigen*	4.04	Cell adhesion
NM_001614.2	ACTG1	*Actin, γ 1*	35.44	Structural constituent of cytoskeleton

Data indicate gene expression changes of S5 over ren transduced cells with a fold change >2 ($p < .05$).
Abbreviations: HSC, hematopoietic stem cell; ren, Renilla RNAi; S5, STAT5 RNAi; signal transducer and activator of transcription 5 RNAi.

Discussion

The present study demonstrates that the STAT5 expression level is a critical factor that determines erythroid versus megakaryocytic lineage fate. Reduction in STAT5 expression promoted lineage commitment to the megakaryocytic lineage at the expense of the erythroid lineage. Reversely, overexpression of activated STAT5 enhanced expansion of the erythroid compartment, whereas the generation of $CD41^+/CD42b^+$ megakaryocytic cells was impaired.

Our previous studies with STAT5 knockdown in human HSCs have recently shown that STAT5 activation is a critical factor at different levels in the hematopoietic system.[18] Reduced STAT5 expression in CB $CD34^+$ cells causes reduced numbers of stem cells and progenitors, which was partly attributed to changes in the cell cycle status.[18] The present study demonstrates that STAT5 is also a critical factor for the lineage commitment to the erythroid and MK lineage. With $CD34^+$ cells, as well as progenitor cells sorted on the basis of CD110, CD45RA, and CD41 expression, it was shown that downregulation of STAT5 changes the balance between the erythroid and megakaryocytic commitment in favor of the MK lineage. A higher number of CFU-MK was noticed in conjunction with an enhanced differentiation along the megakaryocytic lineage. Reversely, expression of activated STAT5 resulted in an induction of erythroid commitment, in line with our previous observations.[20;21] Not only was the strong induction of erythropoiesis observed by overexpression of the STAT5A(1*6) mutant, but similar results have been obtained using the single mutant STAT5A (S711F) or by using fusion proteins of wild-type STAT5A and STAT5B with the

ligand-binding domain of the estrogen receptor, whereby STAT5 activity is induced by administration of 4-hydroxytamoxifen (A.T.J. Wierenga et al., manuscript submitted for publication).

Recently, Buet et al. demonstrated that megakaryocytic progenitors can also be reprogrammed into erythroid progenitors by overexpression of $p210^{Bcr\text{-}Abl}$. [26] The number of CD41- and CD42-positive cells was strongly reduced upon expression of $p210^{Bcr\text{-}Abl}$, whereas the number of cells expressing the erythroid marker GPA was strongly enhanced, in line with our observations using overexpression of activated STAT5. Thus, as STAT5 is one of the main signaling components downstream of $p210^{Bcr\text{-}Abl}$, it is conceivable that the erythroid lineage conversion at the expense of megakaryocyte development involves $p210^{Bcr\text{-}Abl}$-induced STAT5 activity as well. Fli1 was identified as one of the main targets that was downmodulated by $p210^{Bcr\text{-}Abl}$. [26] Whether Fli1 downmodulation is also a prerequisite for STAT5-induced erythropoiesis remains to be elucidated, but our preliminary data do indicate that Fli1 expression is reduced upon STAT5 activation in human cord blood $CD34^+$ cells (unpublished observations).

A number of genes have been identified that have a crucial function in the switch of MEP to the erythroid or MK lineage. GATA1 is indispensable for the differentiation of MEPs toward terminally differentiated erythrocytes, as maturation is arrested at an early proerythroblast-like stage in GATA1-deficient embryos, and GATA1-deficient mice die between embryonic days 10.5 and 11.5 of gestation because of severe anemia [6]. Various studies have shown that reduced expression of GATA-1 promotes the megakaryocytic development. In a megakaryocyte lineage-specific knockout model, it has been shown that depletion of GATA1 in primary megakaryocytes results in hyperproliferation and expansion of the megakaryocytic compartment,

although maturation toward mature platelets was impaired, finally resulting in myelofibrosis.[8;27] Our microarray analysis and Q-PCR data indicated that GATA1 expression is reduced upon depletion of STAT5, whereas introduction of activated STAT5 in human $CD34^+$ cells resulted in an increase in GATA1 expression. These data suggest that the STAT5-induced change in lineage fate determination toward erythroid development might, at least in part, be mediated by changes in GATA1 expression. Megakaryopoiesis induced by downmodulation of STAT5 coincided with reduced levels of Bcl-Xl mRNA, which might be in line with previous data, indicating that megakaryopoiesis is impaired or delayed by overexpression of Bxl-Xl.[28;29] STAT5-induced erythroid commitment was further demonstrated by upregulation of erythroid-specific genes, such as glycophorin A, CD36, hemoglobin α, and hemoglobin γ, in line with our previously published observations.[20] Upon downmodulation of STAT5, many of these erythroid-specific genes were reversibly downregulated as well (including CD36; hemoglobins α, β, ε, and γ; and glycophorin), further strengthening the view that STAT5 is one of the key denominators that determine lineage commitment from the MEP, and it will be challenging to determine in future studies whether these STAT5-mediated changes in erythroid-specific gene expression are all mediated via GATA1.

Our modified progenitor sorting protocol allows further elucidation of molecular mechanisms involved in lineage fate decisions from the MEP into megakaryocytic or erythroid progeny. Efficient separation of myeloid and megakaryocyte/erythrocyte progenitors on the basis of CD45RA and TpoR expression was recently demonstrated by Edvardsson et al. [22] as a modification to the original protocol proposed by Manz et al. [23], in which

CD45RA and CD123 were used. We now included CD41 in our sorting procedures as well and could efficiently isolate megakaryocytic ($CD34^+/CD110^+/CD45RA^-/CD41^+$) and erythroid ($CD34^+/CD110^+/CD45RA^-/CD41^-$) progenitors. Whether our sorting procedures using CD110 and CD41 are also valid for bone marrow cells remains to be elucidated, as one previous report suggested that expression of these markers does not indicate commitment to the megakaryocyte lineage in bone marrow cells [30], in contrast to our findings. Another study in mice indicated that CD9 might also be a useful marker for isolating megakaryocytic progenitors.[31] These cells also coexpressed CD41, and it will be of interest to determine whether CD9 will aid in further purification of megakaryocyte progenitors from human populations as well. Our RNAi studies indicate that depletion of STAT5 from CMP, Ery, and MK populations results in an increase in megakaryocyte progenitors and a decrease in erythroid progenitors, whereas no changes were observed in the GM compartment.

So far, constitutive STAT5 activation has especially been linked to the expansion of the erythroid and MK compartment. V617F JAK2 mutations have recently been identified as an important genetic marker for PV and ET.[11-15] In 80%-90% of PV patients, the JAK2 mutation can be demonstrated, compared with approximately 50% of patients with ET and idiopathic myelofibrosis (IM). A study by Teofili et al. demonstrated recently that the concomitant expression of phosphorylated STAT5 (pSTAT5) by the affected bone marrow cells differs significantly between the separate disorders.[16] Uniformly increased pSTAT5 was demonstrated in PV, whereas reduced pSTAT5 was shown in ET and in patients with IM. This variability in pSTAT5 expression could not be related to the

predominance of the myeloid or erythroid lineage. Apparently, the downstream targets of the JAK2 mutation are cell type-dependently affected and modulated. What is remarkable is the finding that in both disorders in which the megakaryocytic lineage is predominantly affected, a reduced expression of pSTAT5 was observed. These findings are in line with the results of the present study, which demonstrates a critical role for STAT5 expression levels in the commitment toward erythroid and megakaryocytic lineages.

Our data presented here indicate that we can utilize the thrombopoietin receptor c-Mpl in combination with the megakaryocytic marker CD41 to optimize progenitor sorting procedures to further subfractionate the MEP ($CD34^+CD110^+CD45RA^-$) into erythroid (Ery: $CD34^+CD110^+CD45RA^-CD41^-$) and megakaryocytic (Mk: $CD34^+CD110^+CD45RA^-CD41^+$) progenitors from peripheral blood (PB). Furthermore, we have identified STAT5 as a critical denominator that determined lineage commitment between erythroid and megakaryocytic cell fates. Depletion of STAT5 from PB $CD34^+$ cells by a lentiviral RNAi approach in the presence of TPO and SCF resulted in an increase in CFU-Mk while BFU-E were decreased. Activation of STAT5A resulted in the onset of erythropoiesis at the expense of megakaryopoiesis.

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Chapter 5

$KRAS^{G12V}$ enhances proliferation and initiates myelomonocytic differentiation in human stem/progenitor cells via intrinsic and extrinsic pathways

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Submitted for publication

Summary

In human hematopoietic malignancies, *RAS* mutations are frequently observed. Yet, little is known about signal transduction pathways that mediate KRAS-induced phenotypes in human $CD34^+$ stem/progenitor cells. When cultured on bone marrow stroma, we observed that $KRAS^{G12V}$–transduced cord blood (CB) $CD34^+$ cells displayed a strong proliferative advantage over control cells, which coincided with increased early cobblestone (CAFC) formation and induction of myelomonocytic differentiation. However, the $KRAS^{G12V}$-induced proliferative advantage was transient. By week three no progenitors remained in $KRAS^{G12V}$-transduced cultures and cells were all terminally differentiated into monocytes/macrophages. In line with these results, LTC-IC frequencies were strongly reduced. Both the ERK and p38 MAPK pathways, but not JNK, were activated by $KRAS^{G12V}$ and we observed that proliferation and CAFC formation were mediated via ERK, while differentiation was predominantly mediated via p38. Interestingly, we observed that $KRAS^{G12V}$-induced proliferation and CAFC formation, but not differentiation, were largely mediated via secreted factors, since these phenotypes could be recapitulated by treating non-transduced cells with conditioned medium harvested from $KRAS^{G12V}$-transduced cultures. Multiplex cytokine arrays and genome-wide gene expression profiling were performed in order to gain further insight into the mechanisms by which oncogenic $KRAS^{G12V}$ can contribute to the process of leukemic transformation.

Introduction

RAS proteins are small GTPases that control multiple cellular functions like survival, proliferation, differentiation, and cytoskeletal rearrangement.[1;2] Activating mutations in the *RAS* genes, frequently in codons 12, 13, and 61 prevent the hydrolysis of RAS-GTP and result in constitutive activation of the RAS proteins.[2] Three *RAS* genes exist, *NRAS, KRAS* and *HRAS*, and mutations in all of these have been found in large proportion of solid tumors including cancer of the pancreas [3;4], thymus [5], colon [6;7], skin [8], or lung[9;10]. In myeloid leukemias, activating mutations have been found in *NRAS* and *KRAS* genes, in particular in acute myeloid leukemia (AML), chronic myelomonocytic leukemia (CMML), and juvenile myelomonocytic leukemia (JMML).[11-13]

To gain insight into the role of KRAS, HRAS, and NRAS proteins in development and malignant transformation many different model systems have been generated. *HRas* and *NRas* double knockout mice displayed normal development and did not show any abnormalities during their life time. However, *KRas* knockout mice die between embryonic day 12.5 and the term of gestation due to liver problems and anemia.[14] In addition *KRas* knockout embryos displayed increased cell death of motor neurons in the medulla and the cervical spinal cord.[15] These results show that *KRas* gene function is essential for normal mouse development, especially for the hematopoietic and central nervous system; while *NRas* and *HRas* gene functions are dispensable.[16]

Overexpression of oncogenic RAS was studied extensively as well to elucidate the role of RAS in cancer development. Overexpression of

$HRAS^{G12V}$ in human or mouse fibroblast resulted in a permanent G1 arrest which was induced by accumulation of p53 and p16. Inactivation of either p53 or p16 prevented RAS-induced growth arrest in these cells.[17] When the effect of $HRAS^{G12V}$ was studied in erythroid cell differentiation it became clear that $HRAS^{G12V}$ mainly blocks terminal erythroid differentiation and it does not induce apoptosis.[18] In addition, $KRAS^{G12V}$ overexpression from its endogenous promoter in primary mouse erythroid progenitors induced a terminal differentiation block and upon Epo stimulation the downstream target signaling pathways were hyperactivated.[19]

The leukemic potential of KRas, HRas and NRas was compared using the same transplantation model. Mouse bone marrow cells were transduced with retroviral constructs and transplanted into sub-lethally irradiated mice. It was found that all of the three oncogenes had the potential to induce myeloid leukemias but their leukemic potential and the phenotype of the disease was different. NRas caused either a CMML- or AML-like disease in the transplanted mice while KRas-transduced BM cells initiated a CMML-like disease. Animals transplanted with HRas-transduced cells developed an AML-like disease similar to NRas, but in the case of HRas the invasiveness of the tumor was higher and the latency of the disease was shorter.[20]

Transgenic models have been established as well. By using the Mx1-Cre, LSL-$KRas^{G12D}$ mouse model it was observed that overexpression of $KRas^{G12D}$ induced a fatal monocytic myeloproliferative disease in the mice which was similar to CMML and JMML. When signaling pathways were investigated in oncogenic KRas-overexpressing cells it was found that p-Stat5, p-Erk and p-S6 levels were abnormal.[21] Sabnis et al. investigated the effect of $KRas^{G12D}$ on murine hematopoietic stem and progenitor cells and they found that $KRas^{G12D}$ induced a strong proliferative advantage,

increased the fraction of proliferating HSCs, and initiated T-lineage leukemia/lymphoma which was associated with secondary Notch1 mutations. They concluded that MPD-initiating activity was restricted to HSCs in $KRas^{G12D}$ mice and that cooperating mutations appear during cancer progression.[22]

Studies to elucidate the role of RAS mutations in human hematopoietic cells have been performed less frequently. Human CB $CD34^+$ cells transduced with $NRAS^{G13C}$ showed increased expansion in MS5 cocultures and increased myeloid differentiation. Transplantation of $NRAS^{G13C}$-transduced CB $CD34^+$ cells in NOD/SCID mice revealed an increased bone marrow engraftment and higher number of myeloid cells.[23] When c-DNA microarray analysis was performed upregulation of genes encoding cytokines and cycling-dependent kinase inhibitors p16 and p21 were found.

Here, we describe that overexpression of $KRAS^{G12V}$ in human $CD34^+$ CB cells induces a strong proliferative advantage coinciding with the formation of early cobblestones on bone marrow stroma. This enhanced proliferation was however transient, since after three weeks of culture no progenitors remained and all cells terminally differentiated along the myelomonocytic lineage. The involvement of ERK and p38 MAPK pathways in these phenotypes could be dissected, and interestingly we observed that $KRAS^{G12V}$-induced proliferation and CAFC formation, but not differentiation, were mediated at least in part via secreted factors.

Materials and methods

Cell cultures and cell lines

Neonatal CB was collected from healthy full-term pregnancies after informed consent from the obstetrics departments of the Martini Hospital Groningen and the University Medical Center Groningen (UMCG) in The Netherlands. The protocol was approved by the Medical Ethical Committee of the UMCG. Mononuclear cells were isolated using Lymphocyte Separation Medium (PAA Laboratories, Coble, Germany) and $CD34^+$ cells were isolated using the Mini-MACS Separation System (Miltenyi Biotec, Amsterdam, The Netherlands). For MS5 cocultures and LTC-IC assays α-modified medium essential media (Fisher Scientific Europe, Emergo, The Netherlands) supplemented with heat-inactivated 12.5% fetal calf serum (Lonza, Leusden, The Netherlands) and heat-inactivated 12.5% horse serum (Invitrogen, Breda, The Netherlands), penicillin and streptomycin (all from PAA Laboratories), 57.2 μM β-mercaptoethanol (Merck Sharp & Dohme BV, Haarlem, The Netherlands) and 1 μM hydrocortisone (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) so called "Gartner's medium" was used. The MEK inhibitor U0126 was from Promega Benelux B.V. Leiden, The Netherlands, the JNK inhibitor SP600125, and the p38 inhibitor SB203580 were obtained from VWR International, Roden, The Netherlands and were used in concentrations of 5 μM.

Conditioned medium was collected from MS5 cocultures initiated with either MiNR1 control or KRAS-G12V transduced $CD34^+$ CB cells. After one week of coculture medium was harvested and filtered with 0.45-mm

filters (Millipore B.V., Amsterdam Zuidoost, The Netherlands). The filtered medium was aliquoted and stored at -80°C.

For CFC assays $CD34^+$ cells or suspension cells from cocultures (1-5 × 10^3 cells) were plated in duplicate in 35-mm tissue culture dishes containing 1 mL assay medium consisting of methylcellulose (StemCell Technologies, Grenoble, France) supplemented with IMDM (PAA Laboratories, Coble, Germany), 20 ng/mL IL-3, 20 ng/mL IL-6 (both of them from Gist-Brocades, Delft, The Netherlands), 20 ng/mL G-CSF (Rhone-Poulenc Rorer, Amstelveen, The Netherlands), 20 ng/mL c-kit ligand (Amgen, Thousand Oaks, USA), and 6 U/mL erythropoietin (Janssen-Cilag B.V., Tilburg, The Netherlands). After 14 days of culturing colony-forming unit granulocyte-macrophage CFU-GM, burst-forming unit erythroid (BFU-E) and colony-forming unit granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) were scored. For LTC-IC limiting dilution assays cells were plated in the range of 5-1000 cells per well in a 96 well plate in Gartner's medium on MS5. At week five methylcellulose supplemented with the same cytokines as in the CFC assay was added to the wells. Two weeks later wells containing CFCs were scored as positive or negative and LTC-IC frequencies were calculated using ELDA.[24]

Retroviral production and transduction

Stable PG13 MiNR1 control and PG13 KRAS-G12V retroviral producers were cultured in DMEM (Lonza, Leusden, The Netherlands) supplemented with heat-inactivated 10% fetal calf serum and penicillin/streptomycin. Viral particles for retroviral transduction were collected after 8-12 hours of culturing virus producers in hematopoietic progenitor cell growth medium (HPGM) (Lonza, Leusden, The Netherlands). Right before the transduction

supernatants were collected and filtered through 0.45-mm filters. 48 hours before the first transduction round CB $CD34^+$ cells were pre-stimulated in HPGM supplemented with stem cell factor (SCF; 100 ng/mL), Flt3 ligand (Flt3L; 100 ng/mL; both from Amgen, Thousand Oaks, USA), and thrombopoietin (TPO; 100 ng/mL; Kirin, Japan). Pre-stimulated CB cells were transduced on retronectin-coated plates, (retronectin from Lucron Bioproducts B.V, Gennep, The Netherlands) in 3 consecutive rounds of 8 and 12 hours with retroviral supernatant supplemented with the same cytokines and 4 μg/mL Polybrene (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). After transduction, NGFR-positive cells were sorted on a MoFlo (Dako Cytomation, Carpinteria, CA, USA).

Flow cytometry analysis and cell sorting

Antibodies for FACS analysis and for cell sorting were the following: CD34, CD15, CD235a, CD271(NGFR) from BD Bioscience Breda The Netherlands and CD11b, CD14 antibodies were from BioLegend Europe B.V. Uithoorn, The Netherlands. Cell sorting of transduced cells was performed on the basis of NGFR and the sort was performed on a MoFlo and FACS analysis was performed on a BD FACS Calibur.

Cytokine Multiplex array

Cytokine levels were determined by the Human 25-Plex panel (Invitrogen, Breda, The Netherlands). The procedure was performed according to the manufacturer's recommendations using conditioned medium from day 5.

Immunoblotting and cytospins

Cytospins were stained with May-Grunwald-Giemsa staining and images were taken with an Leica DM 3000 microscope (Leica Geosystems B.V., Wateringen, The Netherlands) using a 40x1.3 numeric aperture objective. For Western blot analysis we used control and KRAS G12V-transduced CB CD34$^+$ cells which were cultured in MS5 cocultures for one week. Cells were lysed in Laemmli sample buffer and loaded on 10% SDS acrylamide gel. Proteins were transferred to PVDF membranes (Millipore, Etten Leur, The Netherlands) using semidry electroblotting. KRAS antibody was obtained from Tebu Bio BV, Oosterhout The Netherlands, p-JNK, and pERK1/2 antibodies were from Cell Signaling Technology. Secondary antibodies were purchased from Dako Cytomation (Dako Cytomation, Glostrup, Denmark) and they were used in 1:3000 dilutions.

mRNA analysis

The RNeasy kit (Qiagen Benelux B.V. Venlo, The Netherlands) was used for total RNA isolation. The procedure was performed according to the manufacturer's recommendations from transduced and sorted CB CD34$^+$ cells. For genome-wide expression analysis Illumina (Illumina, Inc., San Diego, CA) BeadChip arrays (Sentrix Human-6; 46,000-probe sets) were used. Hybridization with the arrays was performed according to the manufacturer's instructions. Data were analyzed using the BeadStudio v3 gene expression module (Illumina, Inc.) and Genespring (Agilent, Amstelveen, The Netherlands).

Statistical analysis

All values are expressed as means ± SE. Student's *t* test was used for all other comparisons. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Expression of $KRAS^{G12V}$ in human CB $CD34^+$ cells results in a transient proliferative advantage, the formation of CAFCs and myelomonocytic differentiation.

To study $KRAS^{G12V}$-induced phenotypes in human stem and progenitor cells we inserted the $KRAS^{G12V}$ gene into the murine stem cell virus (MSCV) retroviral expression vector which contained an encephalomyelocarditis virus-derived internal ribosomal entry site (IRES2) in front of the truncated neural growth factor receptor (MiNR1 vector, Figure 1A). Stable PG13 virus producer cell lines were generated and CB $CD34^+$ cells were transduced. After transduction cells were sorted and the overexpression level of KRAS was determined by Western blot analysis. KRAS protein expression was increased approximately four-fold in the $KRAS^{G12V}$-transduced cells compared to control MiNR1-transduced cells (Figure 1B). Transduced and sorted MiNR1 control and $KRAS^{G12V}$ cells were used to initiate MS5 cocultures and expansion and differentiation were analyzed weekly. These experiments revealed that KRAS overexpression in CB $CD34^+$ cells induced a massive proliferation within the first weeks but after the second week $KRAS^{G12V}$-transduced cells stopped proliferating. The strong initial expansion in the $KRAS^{G12V}$-transduced cultures coincided with

the formation of early cobblestones with high frequency within five days after plating (Figure 1D).

To evaluate the changes in stem cell frequencies induced by $KRAS^{G12V}$, LTC-IC assays were performed in limiting dilution. These experiments indicated that LTC-IC frequencies decreased dramatically upon $KRAS^{G12V}$ overexpression (1/1768 in MiNR1 versus 1/22760 in $KRAS^{G12V}$, Figure 1E). CFC assays revealed that progenitor frequencies were increased 6-fold in KRAS-transduced cells within 1 week after plating on MS5, but this increase was transient. Progenitors were exhausted by week 3 since no CFCs were observed in methylcellulose assays. Also, less BFU-Es were observed in the $KRAS^{G12V}$-transduced cultures compared to MiNR1 controls at week 1 (Figure 1F). To determine the differentiation profile in the cocultures cell surface markers were measured by FACS analysis. These experiments showed increased CD14 and CD11b expression in the $KRAS^{G12V}$-transduced cultures compared to MiNR1 controls (Figure 1G). Cytospins from suspension cells of the cocultures confirmed these data and indicated that $KRAS^{G12V}$-transduced cells were all terminally differentiated along the myelomonocytic lineage by week 3 (Figure 1H).

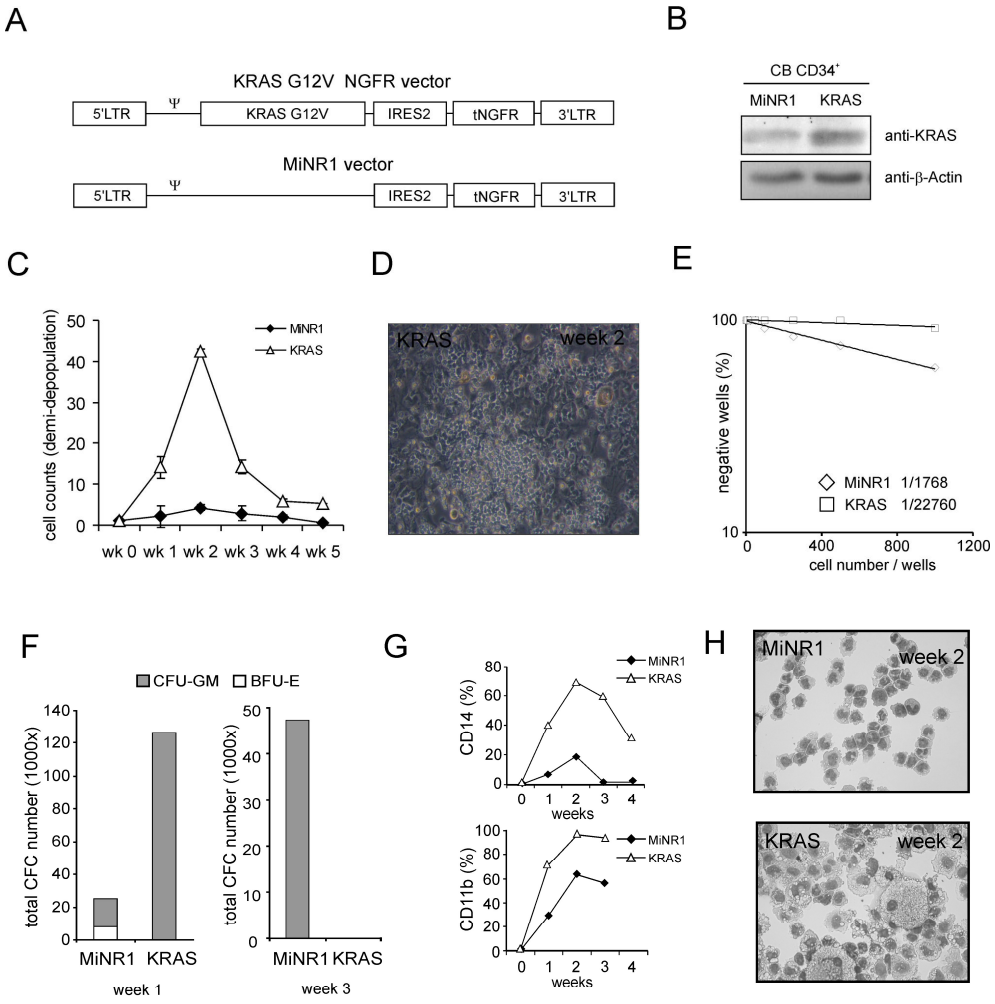


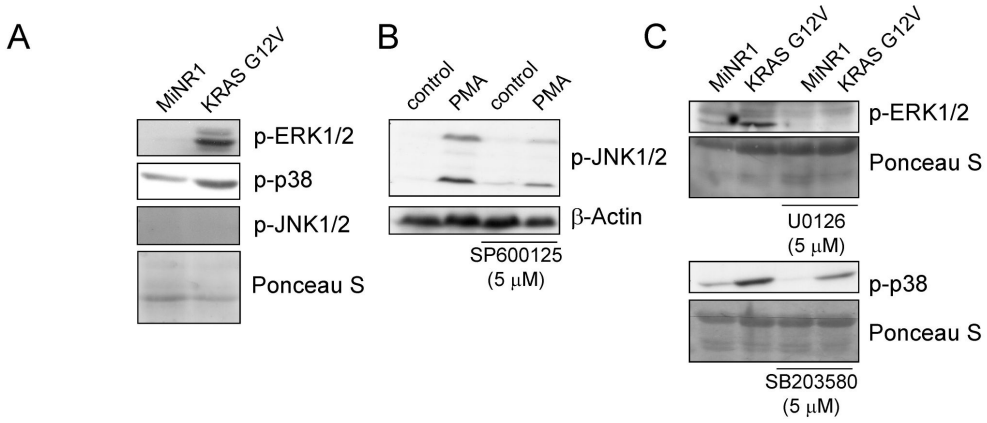
Figure 1. KRAS^{G12V} induces a transient growth advantage, early CAFC and enhanced monocyte/macrophage differentiation. A, Schematic representation of the retroviral vectors used in this study. B, Cord Blood (CB) CD34^{+}cells were transduced with MiNR1 or KRAS^{G12V} vectors, cells were harvested after 48 hours and KRAS expression levels were measured by Western blotting. C, CB CD34^{+} cells were transduced with MiNR1 or KRAS^{G12V} vectors and cells were cultured in long-term MS5 co-cultures. Data shows cell counts of weekly demidepopulation. The average of three independent experiments is shown. D, To determine stem cell frequencies LTC-IC assays in limiting dilution were performed on MiNR1 and KRAS^{G12V} transduced CB CD34^{+} cells. E, Cobblestone Area

Forming Cells (CAFCs) induced by KRAS^{G12V} in MS5 cocultures at week 2. F, From each culture at week 1 and week 3 2000 cells were plated in CFC assays in methylcellulose, colonies were evaluated after two weeks and total CFC numbers were calculated based on the cell number in the cocultures. G, FACS analysis on suspension cells from cocultures indicated monocytic differentiation in KRAS^{G12V} cocultures. H, MGG-stained cytospins from week 2.

Inhibition of ERK impairs proliferation while inhibition of p38 impairs proliferation and differentiation in CD34$^+$CB cells transduced with KRAS^{G12V}.

To determine which signaling pathways could be mediating KRAS^{G12V}-induced phenotypes we first determined which MAPK signal transduction pathways were activated downstream of KRAS^{G12V} by Western blot analysis. These experiments revealed that both the ERK/MAPK and p38/MAPK pathways were activated by KRAS^{G12V} in CB CD34$^+$ cells resulting in enhanced phosphorylation levels of ERK1/2 and p38 (Figure 2A). JNK was not activated by KRAS^{G12V}, while PMA did induce phosphorylation of JNK (Figure 2A and B). Next, specific inhibitors against MEK (U0126), p38 (SB203580) or JNK (SP600125) were used in order to dissect the involvement of MAPK pathways in KRAS^{G12V}-induced phenotypes. Western blot analysis confirmed that the inhibitors effectively downmodulated the activity of the designated pathways (Figure 2B and C). To determine the changes in growth, cobblestone formation and differentiation CB CD34$^+$ cells were transduced with either control MiNR1 or KRAS^{G12V} vectors. Sorted cells were used to initiate MS5 cocultures which were analyzed for 5 weeks. Inhibition of MEK by U0126 reduced proliferation, both of KRAS^{G12V} as well as MiNR1-transduced cells (Figure 2D). Inhibition of p38 using SB203580 also impaired the expansion of

$KRAS^{G12V}$-transduced cells, but to a lesser extent compared to cells treated with the MEK inhibitor (Figure 2E). Surprisingly, MiNR1 control cells treated with the p38 inhibitor proliferated slightly better compared to controls (Figure 2E). As expected, no effects were observed on the growth of either MiNR1 control cells or $KRAS^{G12V}$ cells treated with the JNK inhibitor SP600125 (data not shown). FACS analysis was used to determine the effects of ERK and p38 on $KRAS^{G12V}$-induced differentiation. As observed earlier, $KRAS^{G12V}$ enhanced the differentiation towards CD11b and CD14 positive myelomonocytic cells, which was not affected by inhibition of MEK (Figure 2F). In contrast, inhibition of p38 by using the inhibitor SB203580 did significantly impair $KRAS^{G12V}$-induced myelomonocytic differentiation (Figure 2G).



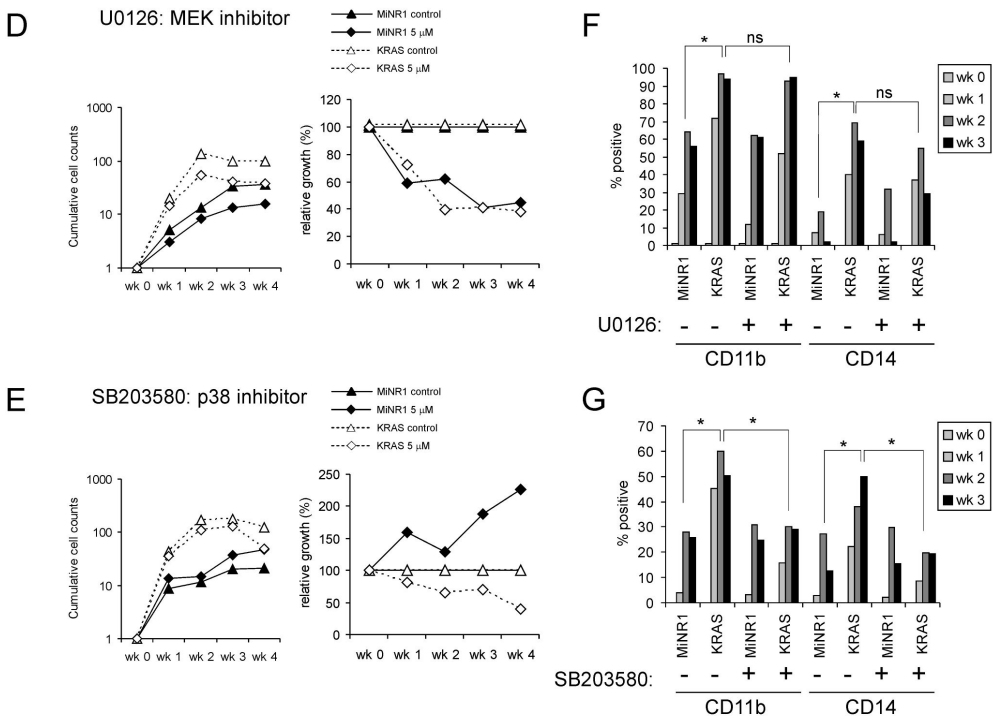


Figure 2. Inhibition of ERK impairs proliferation while inhibition of p38 impairs proliferation and differentiation in CB CD34$^+$ cells transduced with KRAS^{G12V}. A, Western blots of MiNR1 and KRAS^{G12V}-transduced CB CD34$^+$ cells. B, UT7 cells were treated with PMA for 15 min, and cells were pretreated with the JNK inhibitor SP600125 as indicated after which lysates were prepared for Western blotting. C, Western blots on transduced cells as in A, but now cells were pretreated with the MEK inhibitor U0126 or the p38 inhibitor (SB203580) as indicated. D, Proliferation of MiNR1 or KRAS^{G12V} cells in MS5 coculture in the presence of MEK inhibitor (U0126). Cultures were demi-depopulated weekly and cumulative cell counts are shown. In the right panels, the relative effects of the inhibitor are shown whereby the untreated MiNR1 and KRAS^{G12V} cell counts were normalized to 100% every week. E, As in D, but now MS5 cocultures were performed in the presence of p38 inhibitor (SB203580). F, FACS analysis of cultures described in E. G, FACS analysis of cultures described in E.

Conditioned medium from KRAS^{G12V}-transduced cells is sufficient to induce proliferation and formation of early CAFCs in CB CD34^{+} cells.

To our surprise, when MS5 cocultures were initiated with non-sorted MiNR1 control and KRAS^{G12V} transduced CB CD34^{+} cells, we observed that the non-transduced cells in KRAS^{G12V}-initiated cocultures displayed a proliferative advantage as compared to non-transduced cells in MiNR1-initiated control cocultures. A representative experiment is shown in Figure 3A, using MiNR1 and KRAS^{G12V}-transduced CB cells with initial transduction efficiencies of 52% and 25%, respectively. Cocultures were weekly demi-depopulated, expansion and differentiation of NGFR^{+} and NGFR^{-} cells were monitored, and cumulative expansion data is shown in Figure 3A. NGFR^{+} KRAS^{G12V}-transduced cells displayed a strong proliferative advantage over MiNR1 as observed previously. Unexpectedly, the NGFR^{-} cells in KRAS^{G12V}-transduced cocultures also displayed a proliferative advantage over NGFR^{-} cells from MiNR1 control cocultures with almost similar kinetics as NGF^{+} KRAS^{G12V}-transduced cells (Figure 3A, lower panel). In contrast, the myelomonocytic differentiation induced by KRAS^{G12V} was only observed in transduced cells, and not in non-transduced NGFR^{-} cells in these cocultures.

To further substantiate these findings, conditioned medium (CM) was harvested from KRAS^{G12V} and MiNR1-transduced CB CD34^{+} cells, which was then used in MS5 cocultures initiated with freshly isolated non-transduced CB CD34^{+} cells. As shown in figure 3C, the KRAS^{G12V} CM was sufficient to induce a robust proliferative advantage compared to cells treated with MiNR1 CM. The observed proliferative advantage was also much less transient as compared to directly transduced KRAS^{G12V} transduced CB CD34^{+} cells. The proliferative advantage coincided with

enhanced progenitor frequencies as determined by CFC assays, which persisted for over 6 weeks of coculture (Figure 3D). Also, CAFCs were formed within 2 weeks of coculture of CB $CD34^+$ cells in the presence of $KRAS^{G12V}$ CM, which persisted throughout the culture period (Figure 3E). In line with what was observed in the non-sorted MS5 coculture experiments, no pronounced acceleration of myelomonocytic differentiation was observed in cultures treated with $KRAS^{G12V}$ CM and morphological analysis (Figure 3F) as well as analysis by FACS (Figure 3G) revealed normal myeloid differentiation. Thus, these data indicate that a $KRAS^{G12V}$ induced secreted factor strongly contributes to expansion and CAFC phenotypes, while the induction of myelomonocytic differentiation is induced via intrinsic pathways.

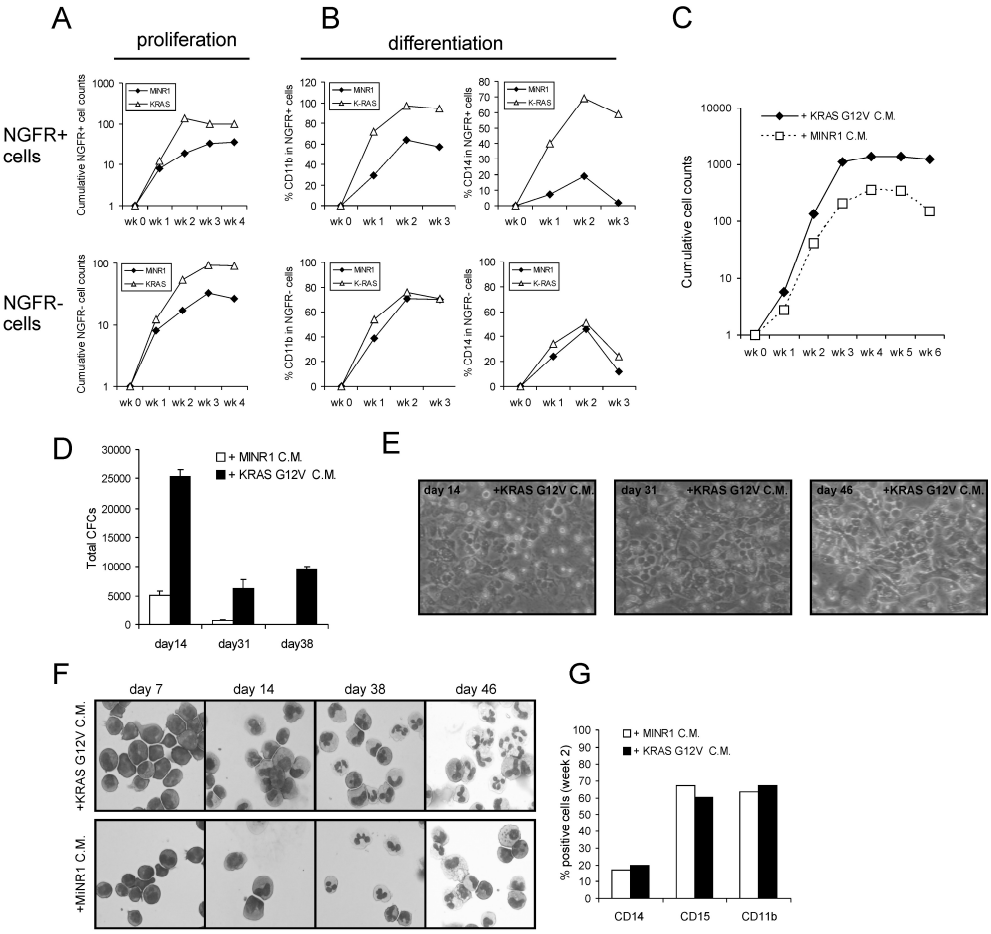


Figure 3. Conditioned medium from KRAS^{G12V}-transduced cells induces a proliferative advantage and the formation of CAFCs but not myelomonocytic differentiation in freshly isolated CB CD34^{+} cells.

A, MS5 cocultures were initiated with non-sorted, MiNR1 and KRAS^{G12V}-transduced CB CD34^{+} cells. The cumulative expansion of NGFR^{+} as well as NGFR^{-} populations is shown. B, Experiment as in A, but now cultures were analyzed by FACS. C, Conditioned medium was collected from MS5 cocultures initiated with MiNR1 and KRAS^{G12V}-transduced CB CD34^{+} cells. Freshly isolated CB CD34^{+} cells were cultured on MS5 in the presence of MiNR1 and KRAS^{G12V} CM as indicated. Cumulative cell counts are shown. D, as in C, but now suspension cells were analyzed for progenitors in CFC assays in methylcellulose. E,

representative images of CAFCs of cultures described in C. F, representative cytospins stained with MGG from suspension cells of cultures described in C. G, FACS data of cultures described in C.

Identification of KRAS^{G12V} target genes in human CB CD34$^+$ cells.

To identify KRAS^{G12V} target genes a genome-wide gene expression profiling study was performed using Illumina BeadChip arrays. CB CD34$^+$ cells were transduced with KRAS^{G12V} and MiNR1 control vectors and transduced cells were sorted. RNA was isolated from each sorted population after 24 hours and the RNA was used for hybridization with Illumina Bead Chip arrays. 2258 differentially expressed genes (>2-fold) upon KRAS overexpression were identified, of which 1167 genes were upregulated and 1091 were downregulated (Supplemental Table 1). Some of the upregulated genes are shown in Table 1, and this list included genes encoding secreted chemokine/cytokine members such as CCL2, CCL5, IL8, IGFBP5, TNF and VEGFA. A number of these genes have been previously identified as targets of MEK activation as well.[25] Furthermore, various transcriptional regulators, including CITED2, FOSB, HOXB2, HOXB5, GSK3, JUN, ID2, MAFA, MAFB, MEIS3 and TRIB1 were upregulated by KRAS^{G12V}. The expression of a number of cell cycle regulators such as CyclinD1, CyclinD3, p19 and p21 was also induced by KRAS^{G12V}. Gene ontology (GO) analysis revealed that the KRAS^{G12V}-upregulated gene list was enriched for genes associating with differentiation, membrane fraction, adhesion, apoptosis, and regulation of the cell cycle (Table 2). While genes associating with myelomonocytic differentiation such as CD14 were also upregulated, no effects of KRAS^{G12V} were observed on the expression of GM-CSFR, CSFR, CEBPα or PU.1. The list of downregulated genes upon overexpression of KRAS^{G12V} was

enriched for transcriptional regulators as determined by GO analysis (data not shown), and included HOXA5, HOXA9, NMYC, LEF1, as well as many erythroid-associated genes (Supplemental Table 1).

To further verify whether upregulation $KRAS^{G12V}$ target genes encoding chemokines/cytokines indeed resulted in actual secretion of these factors, a 25-plex cytokine array analysis was performed on CM from by $KRAS^{G12V}$ and MiNR1-transduced CB $CD34^{+}$ cells. We were able to confirm that the $KRAS^{G12V}$ secretome included IL8, IL12p40, CCL2,-3,-4, and 5, and IL1RA. These data are summarized in Table 3 and the corresponding gene expression data from the Illumina microarray studies is included as well.

Table 1. Genes upregulated by KRAS^{G12D} in human CB CD34$^+$ cells.

gene name	fold change	definition
CCL2	67,05	Chemokine (C-C motif) ligand 2
CCL23	2,26	Chemokine (C-C motif) ligand 23
CCL5	4,50	Chemokine (C-C motif) ligand 5
CCND1	14,61	Cyclin D1
CCND3	2,24	Cyclin D3
CD14	2,58	CD14
CD38	2,19	CD38
CD96	2,21	CD96
CDKN1A	6,98	Cyclin-dependent kinase inhibitor 1A (p21)
CDKN2D	10	Cyclin-dependent kinase inhibitor 2D (p19)
CITED1	47.97	Cbp/p300-interacting transactivator
ESAM	4,15	Endothelial cell adhesion molecule
FOSB	2,17	FBJ murine osteosarcoma viral oncogene homolog B
FOXO4	2,34	Forkhead box O4
GSK3B	2,17	Glycogen synthase kinase 3 beta
HOXB2	2,93	Homeobox B2
HOXB5	3,49	Homeobox B5
ID2	5,92	Inhibitor of DNA binding 2
IFNA10	2,05	Interferon alpha 10
IGFBP5	348	Insulin-like growth factor binding protein 5
IL21R	2,72	Interleukin 21 receptor
IL8	13,17	Interleukin 8
JUN	7,09	Jun oncogene
JUNB	3,65	Jun B proto-oncogene
MAFA	3,79	v-Maf homolog A
MAFB	33	v-Maf homolog B
MCL1	3,27	Myeloid cell leukemia sequence 1
MAP2K3	3,23	Mitogen-activated protein kinase kinase 3
MEIS3	4,08	Meis homeobox 3
MMP9	92,03	Matrix metallopeptidase 9
NCF4	2.26	Neutrophil cytosolic factor 4, p40phox
TIMP1	7,10	TIMP metallopeptidase inhibitor 1
TNF	4,70	Tumor necrosis factor
TRIB1	4,89	Tribbles homolog 1
VEGFA	3,29	Vascular endothelial growth factor A

Table 2. GO term annotations of KRAS G12V-upregulated genes in CB CD34$^+$ cells

GO term	PValue	FDR
GO:0030154~cell differentiation	1.96E-08	3.75E-05
GO:0005624~membrane fraction	1.16E-06	1.80E-03
hsa04510:Focal adhesion	1.48E-05	1.85E-02
GO:0006915~apoptosis	1.22E-05	2.33E-02
GO:0008219~cell death	1.29E-05	2.47E-02
GO:0048468~cell development	1.60E-05	3.06E-02
GO:0012501~programmed cell death	1.64E-05	3.14E-02
GO:0000074~regulation of progression through cell cycle	8.47E-05	1.62E-01
GO:0051726~regulation of cell cycle	9.68E-05	1.85E-01
IPR014393:Dual specificity protein phosphatase (MAP kinase phosphatase)	1.18E-04	2.27E-01
GO:0030099~myeloid cell differentiation	2.96E-04	5.65E-01
GO:0008283~cell proliferation	3.75E-04	7.16E-01

Table 3. KRAS G12V-induced cytokines and growth factors in human CB CD34$^+$ cells (25-plex multi array and Illumina gene expression profiling)

	25-plex cytokine array		Illumina data	
Symbol	**MiNR1 (pg/ml)**	**KRAS (pg/ml)**	**MiNR1**	**KRAS**
IL-1B	1,00	6,51	6897	10891
IL-1RA	27,93	3757,36	346	664
IL-8	1,50	10010,62	1669	29251
IL-12p40	2,00	199,83	nd	nd
MIP-1a (CCL3)	4,00	27,24	nd	nd
MIP-1b (CCL4)	0,40	88,59	nd	nd
IP-10 (CXCL10)	0,00	60,71	nd	nd
RANTES (CCL5)	2,00	35,04	127	553
MCP-1 (CCL2)	19,41	2145,37	60	4023

Discussion

The effects of KRAS mutations on hematopoiesis have been studied extensively in murine model systems, and the effects on proliferation and myeloid commitment have been analyzed in substantial detail. Yet, KRAS-induced phenotypes in the human system have been poorly described, and little insight has been obtained regarding the molecular mechanisms that are involved. Here, we describe that retroviral introduction of KRAS^{G12V} in human CB CD34$^+$ results in increased proliferation coinciding with the formation of early cobblestone areas underneath the stroma, followed by terminal differentiation along the myelomonocytic lineage. Importantly, we identify the ERK/MAPK pathway as an extrinsic mediator of KRAS^{G12V}-induced phenotypes, whereby a growth advantage as well as the capacity to form CAFCs underneath bone marrow stromal cells can be imposed on both transduced cells as well as on non-transduced cells in a paracrine manner. KRAS^{G12V}-induced myelomonocytic differentiation is predominantly regulated via intrinsic pathways mediated via p38/MAPK.

RAS-induced activation of the ERK/MAPK pathway has been linked to enhanced cell cycle progression in various tissues. Amongst others, this has been associated with an upregulation of cell cycle genes such as CyclinD1,-2, and -3.[25-27] Indeed, we also observed an upregulation of these positive cell cycle regulators in KRAS^{G12V}-transduced CB CD34$^+$ cells. However, besides these direct effects of the RAS-ERK pathway on the regulation of cell cycle progression, our data now indicate that secreted factors that mediate KRAS^{G12V}-induced proliferation in an autocrine/paracrine manner

must exist as well. In untransduced cells in non-sorted cocultures, as well as in freshly isolated CB $CD34^+$ cells that were treated with conditioned medium (CM) harvested from $KRAS^{G12V}$-transduced cells, we observed a striking proliferative advantage over cells that had not been cultured in the presence of $KRAS^{G12V}$-expressing cells or CM. Not only the proliferation was enhanced by CM, also the formation of CAFCs was dramatically increased. Experiments whereby $KRAS^{G12V}$-transduced cells were grown in cytokine-driven cultures revealed that the proliferative advantage of $KRAS^{G12V}$-expressing cells compared to controls was not as dominant as observed in MS5 bone marrow stromal cocultures (data not shown). Thus, these data suggest that the formation of CAFCs plays an important role in the autocrine/paracrine proliferative advantage induced by $KRAS^{G12V}$. Genome-wide gene expression profiling allowed the identification of various chemokines, cytokines and growth factors as target genes of $KRAS^{G12V}$, and a number of these were indeed confirmed as being secreted by $KRAS^{G12V}$-transduced cells as determined by 25-plex cytokine arrays. The most pronounced were CCL2, IL1RA and IL8. In line with our observations, it was recently reported that expression of $KRAS^{G12V}$ in various human cell lines upregulated a number of growth factors and chemokines, including VEGF, IL6, CXCL1 and CXCL8 (IL8).[28] Upregulation of IL8 and a number of these chemokines by activation of the RAS/ERK pathway was reported by others as well.[25;29] Importantly, deletion of CXCR2, the common receptor for chemokines such as IL8 reduced oncogenic RAS-driven tumorigenesis in mice, indicating that RAS-induced secretion of these factors indeed participates in the process of transformation.[28] Moreover it might create a microenvironment that gives the malignant clone a growth advantage over their normal counterpart.

Possibly, a multitude of the KRAS^{G12V}-secreted factors act in collaboration, and our current studies are aimed at gaining further insight into the KRAS^{G12V} secretome. Regardless, it remains remarkable that cells that do not express an oncogene are strongly affected via autocrine signaling provided by neighboring cells that express KRAS^{G12V}. In this respect it is interesting to note that FOSB was also upregulated by KRAS^{G12V} in CB CD34$^+$ cells. Recently, a number of genes were identified in a stem cell activity screen that could enhance repopulation activity in a non-cell-autonomous manner, and one of these was FOS.[30] It will be interesting to determine whether FOS also mediates the expression of KRAS^{G12V}-induced chemokines and growth factors.

KRAS^{G12V}-induced myelomonocytic differentiation was predominantly mediated via p38/MAPK, and not via the ERK/MAPK pathway. Since our experiments using KRAS^{G12V} conditioned medium revealed that only proliferation but not myelomonocytic differentiation was affected, we conclude that the p38/MAPK-mediated differentiation in KRAS^{G12V} cells involves activation of intrinsic pathways. The GO term myeloid cell differentiation was significantly enriched in the list of KRAS^{G12V}-upregulated genes, and it is possible that a number of these genes are activated via the p38/MAPK pathway. In line with previous observations [19;31], we find that activation of RAS impairs erythropoiesis, and the reduction of BFU-Es in KRAS^{G12V}-transduced cells coincided with a reduction in expression of erythroid genes. The JNK pathway does not appear to play a major role in the observed phenotypes since we did not observe phopshorylation of JNK in KRAS^{G12V}-transduced cells and treatment with the JNK inhibitor SP600125 neither affected proliferation or differentiation. Recently, it was reported that HRAS^{G12V} can also induce

phosphorylation of p38.[26] This induction of p38 activity was attributed to $HRAS^{G12V}$-upregulation of reactive oxygen species (ROS) as treatment with the antioxidant diphenyleneiodonium impaired phosphorylation of p38. Furthermore, the induction of ROS associated with enhanced proliferative capacity of $HRAS^{G12V}$-transduced cells and the increase in proliferation could be counteracted by treatment with antioxidants.[26] Whether differentiation of $HRAS^{G12V}$-transduced cells was also affected by treatment with antioxidants is currently unclear. Remarkably, while inhibition of p38 impaired the differentiation, and also to some extent the proliferation of $KRAS^{G12V}$-transduced cells in our studies, we observed that MiNR1 controls cells produced significantly more progeny in bone marrow cocultures. It was reported that ROS-induced p38 activity resulted in stem cell exhaustion and that inactivation of p38 extends the lifespan of HSCs in serial transplantation assays.[32] Possibly, immature HSC/MPPs were better maintained in our MiNR1 cultures treated with SB203580 resulting in the production of more progeny. We do not know whether $KRAS^{G12V}$ also upregulates ROS levels in CB $CD34^+$ cells as was observed in $HRAS^{G12V}$-transduced cells where it was associated with enhanced proliferation [26], but it is well possible that the production of ROS induced by RAS initially results in enhanced proliferation, but that ultimately hyperactivation of p38 in $KRAS^{G12V}$-transduced cells induces exhaustion of the stem cell pool as we indeed observed in our studies.

While RAS mutations are frequently observed in human leukemias, it is remarkable that we do not find long-term stem cell self-renewal or transformation phenotypes in our human CB $CD34^+$ model systems. In line with our observations, it was reported that expression of $NRAS^{G13C}$ enhanced engraftment in NOD-SCID mice, but could not induce

leukemia.[23] Also in mouse models, KRAS mutations were able to generate a myeloproliferative disease, but not leukemia.[21;33;34] Thus, oncogenic RAS might require additional mutations in order to induce overt leukemia, and indeed in a number of model systems it was shown that RAS mutations can effectively cooperate with additional hits.[22;35;36]

The link between RAS mutations and monocytic differentiation is not completely clear in AML. Recently it was shown that AML patients with the CBFβ mutation frequently have alterations in the RAS pathway.[37] Therefore RAS mutations might be linked more to an advantage in growth and survival than to differentiation defects. For example, it was shown previously that activated RASs induces the NF-κB pathway in AML and this mechanism increases the resistance to apoptosis.[38] In CMML, NRAS mutations are frequently noticed suggesting a link between monocytic differentiation and the mutation. However, recently a number of additional mutations have been defined in CMML such as TET2 and ASXL2.[39;40] So far, it is unknown if these mutations maintain the stem cell phenotype and whether the RAS mutation triggers the differentiation program. Furthermore, RAS mutations and MLL rearrangements in B-precursor childhood ALL were closely associated.[41]

In summary, our data demonstrate that overexpression of KRAS^{G12V} in CB CD34$^+$ cells enhances proliferation transiently, increases early cobblestone formation, reduces LTC-IC frequencies and initiates monocytic differentiation. We observed that KRAS^{G12V} activates both ERK and p38MAPK pathways but not JNK in CB CD34$^+$ cells. Activation of the ERK pathway correlated with proliferation which was mediated at least in part via secreted factors that can act in an autocrine/paracrine manner.

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Chapter 6

Summary, discussion and future perspectives

Summary and discussion

Chromosomal translocations or molecular abnormalities in hematopoietic stem cells can disrupt the normal process of self-renewal and differentiation, which can result in malignant transformation. A number of key transcription factors and signaling molecules have been identified that function aberrantly in leukemic cells. In this thesis we investigated the effects of STAT5 and KRAS on human hematopoietic stem cell self-renewal and differentiation, and their potential role in leukemic transformation.

Constitutive activation of STAT5 in $CD34^+$ cord blood cells by retroviral transduction resulted in the upregulation of the membrane protein MUCIN1. Based on this information (**chapter 2)** we investigated the role of MUCIN1 in normal hematopoiesis as well as in primary AML cells. We demonstrated that 10% of the cord blood $CD34^+$ cells were MUCIN1-positive. MUCIN1 mRNA expression was the highest in the $CD34^+/CD38^-$ cell fraction. Experiments with cord blood $CD34^+/MUCIN1^+$ and $CD34^+/MUCIN1^-$ cell populations revealed that stem/progenitor cells reside predominantly in the $CD34^+/MUCIN1^+$ fraction. To further study the role of MUCIN1 in hematopoiesis we stably overexpressed full-length MUCIN1 and a MUCIN1 isoform with a deleted extracellular domain (DTR) in cord blood $CD34^+$ cells. In MS5 coculture assays a two-fold increase in expansion of suspension cells was observed, and LTC-IC frequencies and progenitor numbers were increased upon overexpression of MUCIN1 or DTR.

Next, we aimed to investigate the underlying mechanisms that might be responsible for the MUCIN1-induced phenotypes. It has been published that upon ligand binding, the intracellular cytoplasmic tail of MUCIN1 is cleaved and initiates multiple signalling pathways.[1-3] Chromatin immunoprecipitation (ChIP) assays on MUCIN1-overexpressing cancer cells have shown that the MUCIN1 cytoplasmic tail (CT) can bind to p53 promoter elements indicating that MUCIN1 CT plays role in transcriptional regulation.[3] Another known binding partner of MUCIN1 CT is β-CATENIN which can shuttle to the nucleus and activate target genes.[4] In our model system we measured the nuclear translocation of β-CATENIN and the expression of CYCLIN D1 by qRT-PCR. However, no difference was found between the control, the Mucin1-transduced, or DTR-transduced cells. The NF-κB signalling is an alternative pathway that can be activated by MUCIN1. Ahmad et al. demonstrated that overexpression of MUCIN1 in human carcinoma cells is associated with constitutive activation of NF-κB p65.[5] In our model system the activation of NF-κB p65 was confirmed suggesting that MUCIN1-induced phenotypes involve increased cell survival mechanisms.

Furthermore, we studied whether MUCIN1 overexpression increases the interaction between stromal and hematopoietic cells. It has been shown that the heavily *O*-glycosilated large extracellular tandem repeat (TR) domain of MUCIN1 interacts with intercellular adhesion molecule 1 (ICAM1) and this interaction initiated migration.[6] In addition a study from Regimbald et al. demonstrated that the interaction between MUCIN1 and ICAM1 mediates adhesion between endothelial cells and breast cancer cells.[7] ICAM1 is expressed by stromal cells in the stem cell niche which

could suggest a possible role for ICAM1 in the hematopoietic cell – microenvironment interaction. Indeed, when we overexpressed ICAM1 in MS5 stromal cells, we observed increased LTC-IC frequencies in control cells and even higher LTC-IC frequencies in MUCIN1-overexpressing cells suggesting that the ICAM1-mediated MUCIN1 signaling facilitates increased interaction between hematopoietic and stromal cells.

In AML, we observed also increased MUCIN1 expression in 70% of the AML cases, suggesting that elevated MUCIN1 levels might play a role in regulating the proliferative potential of the immature leukemic compartment as well. The high MUCIN1 expression was observed mainly in the $CD34^+$ AML population but in some cases MUCIN1 was also elevated in the $CD34^-$ population. Since the majority of leukemic stem cells are residing in the $CD34^+$ fraction, the elevated MUCIN1 levels in the $CD34^+$ compartment could play a role in regulating the proliferative capacity of the primitive leukemic compartment and/or might modulate the interaction with the microenvironment.

Thus, our data demonstrate that HSCs as well as $CD34^+$ AML cells are enriched for MUCIN1 expression, and that overexpression of MUCIN1 in cord blood $CD34^+$ cells is sufficient to increase expansion, progenitor numbers, and stem cell frequencies.

STAT5 is widely expressed in the hematopoietic system, including stem and progenitor cells, committed erythroid, myeloid and lymphoid cells. It plays an essential role in normal hematopoiesis as well as in the development of leukemia. Until now, little is known about mechanisms which are regulated by STAT5 in these processes. In **chapter 3,** we studied whether STAT5-imposed long-term self-renewal is exclusively restricted to

the HSC compartment or whether long-term self-renewal can also be imposed on progenitor cells. For this study retroviral expression systems were used where STAT5 was fused to the estrogen receptor ligand binding domain (ER) allowing the induction of STAT5 activity by treatment of cells with 4-hydroxytamoxifen (4-OHT). Human cord blood cells were transduced with control and STAT5-ER retroviral vectors followed by MoFlo sorting into four populations: hematopoietic stem cells (HSC, defined as $CD34^{+}CD38^{low}$), common myeloid progenitors (CMP, $CD34^{+}CD38^{+}CD123^{+}CD45RA^{-}$), granulocyte-macrophage progenitors (GMP, $CD34^{+}CD38^{+}CD123^{+}CD45RA^{+}$) and megakaryocyte-erythroid progenitors (MEP, $CD34^{+}CD38^{+}CD123^{-}CD45RA^{-}$). After sorting, MS5 bone marrow stromal cocultures were initiated and STAT5 activity was induced by 4-OHT. In HSCs, STAT5 overexpression induced a long-term proliferative advantage as well as a significant increase in cobblestone formation. This coincided with elevated levels of colony forming cells (CFCs) that were maintained over 5 weeks. In contrast, STAT5 was unable to induce cobblestone formation in progenitor cocultures and only a transient STAT5-induced increase in cell numbers was observed in cocultures initiated with CMPs and MEPs while GMPs did not expand. These data showed that HSCs but not myeloid or erythroid progenitors are the targets of STAT5-imposed long-term growth. This is in line with the results of previous investigations which showed that constitutively active STAT5 increases self-renewal and replating potential in the $CD34^{+}/CD38^{low}$ cell fraction.[8] In addition, when constitutively active STAT5 was expressed in the $CD34^{-}$ LSK cell population in mice a myeloproliferative disease was induced, which was not observed when activated STAT5 was expressed in

progenitor cells.[9] These data suggest that STAT5 is not capable to reinstall self-renewal in cells that have already lost their self-renewal potential.

Next, we aimed to identify STAT5 target genes which were upregulated in the STAT5 HSC population that were not responsible for erythroid differentiation but rather played a role in STAT5-induced self-renewal and long-term expansion. GATA1 was downmodulated in STAT5-transduced cord blood cells by a lentiviral RNAi approach, which completely abrogated erythropoiesis but maintained enhanced HSC self-renewal. Gene expression profiling was performed on both GATA1 downmodulated STAT5-transduced cord blood cells as well as on STAT5-transduced HSC and progenitor populations. By comparison of the data from the two experiments, 32 GATA1-independent STAT5 target genes were identified in the STAT5 HSC population. One of the most prominent altered genes which was further investigated was hypoxia-induced factor 2 (HIF2α/EPAS1).[10-12] HIF proteins are transcriptional regulators of hypoxia-inducible genes. Under hypoxic conditions the α subunit (HIF1α, HIF2α or HIF3α) is stabilized and binds to the constitutively expressed β subunit. This complex activates hypoxia-inducible genes by binding to hypoxia response elements (HRE).[13] The fact that both HIF1$\alpha^{-/-}$ and HIF2$\alpha^{-/-}$ mouse embryos die suggest that HIF1α and HIF2α can not complement each other functionally.[14-16] HIF1α and HIF2α have 48% of sequence identity and they have many common transcriptional targets such as VEGF, TIE2 and ANG2. However, HIF2α has unique targets as well such as OCT4 or TGFα which suggest that HIF2α might play a role in other mechanisms such as pluripotency.[12] Furthermore, it was recently shown that in solid tumours

mir-210 is regulated by HIF1α but not by HIF2α suggesting further differences between HIF1α and HIF2α fuction.[17]

Interestingly we observed that STAT5 activation in cord blood $CD34^+$ cells induces cMYC and VEGF expression and downmodulation of HIF2α by HIF2α RNAi in STAT5-activated cells reduced the expression of these genes. Furthermore, our data showed that downmodulation of HIF2α in STAT5-activated cord blood cells reduced STAT5-induced proliferation, CFC numbers and LTC-IC frequencies while apoptosis and differentiation were unchanged. These results show that the STAT5-induced long-term phenotypes are at least partially mediated by HIF2α. These data demonstrated that STAT5 activation alters hypoxia inducible genes through HIF2α upregulation that normally is only activated during hypoxic conditions. Using this mechanism the malignant clone might obtain the opportunity to move outside the niche and preserve its more immature properties.

Our microarray experiment revealed that STAT5 target genes in HSCs, CMPs, GMPs, and MEPs did not overlap significantly which suggest that different sets of genes are activated by STAT5 in these different compartments. The GMP population behaved probably the most different from the other populations. GMPs did not expand upon STAT5 activation which was unexpected because STAT5 can be activated by various myeloid growth factors indicating that additional signalling routes have to be stimulated. In addition, the GMP population was the only population which did not show any erythroid differentiation upon constitutive activation of STAT5.

Taking together, our data show that hematopoietic stem cells, but not progenitors are the exclusive target for STAT5-induced long-term self-renewal. Furthermore, we show that HIF2α is a novel STAT5 target gene which plays an important role in STAT5-induced stem cell phenotypes.

In **chapter 4,** we investigated the potential role of STAT5 in erythroid and megakaryocytic lineage development. Our results show that downmodulation of STAT5 in $CD34^+$ peripheral blood stem cells (PBSC) in the presence of thrombopoietin (TPO) and stem cell factor (SCF) resulted in an increase in megakaryocytic progenitors, while erythroid progenitors were decreased. On the other hand, overexpression of activated STAT5A(1*6) mutants severely impaired megakaryocyte development and induced a robust erythroid differentiation. These data indicate that constitutively active STAT5 induces erythroid differentiation while megakaryocyte development is impaired. These data also indicate that, besides affecting the stem cell compartment as we observed in chapter 3, STAT5 can also be involved in fate decisions of progenitor cells. Our data now show that downregulation of STAT5 changes the balance between the erythroid and megakaryocytic commitment and promotes differentiation towards the megakaryocytic lineage. The opposite was observed by overexpression of activated STAT5 which resulted in increased erythroid differentiation in line with our previous observations.[8;18;19]

Our microarray and qRT-PCR data suggest that STAT5-induced lineage commitment might be partially mediated by GATA1 expression since GATA1 expression was reduced in STAT5 downmodulated cells and in STAT5-activated cells, GATA1 levels were increased. These results are

in agreement with published data from the megakaryocyte lineage-specific GATA1 knock out mouse model where GATA1 levels were reduced in megakaryocytes, resulting in expansion of the megakaryocytic compartment, finally resulting in myelofibrosis.[20;21] In addition, our microarray data revealed that downmodulation of STAT5 in normal $CD34^+$ cells reduced Bcl-XL expression which was in line with an earlier study showing that overexpression of Bcl-XL delayed or impaired megakaryopoiesis.[22]

To further study the effect of STAT5 downmodulation on lineage decisions in specific cell compartments we established a sorting procedure based on the publications of Edvardson et al. and Manz et al.[23] In order to distinguish the megakaryocytic from the erythroid compartment we used the cell surface marker CD41. For the isolation of progenitor fractions the following marker combinations were used: common myeloid progenitors (CMP, $CD34^+/CD110^-/CD45RA^-$), granulocyte-macrophage progenitors (GMP, $CD34^+/CD110^-/CD45RA^+$), megakaryocyte-erythroid progenitors (MEP, $CD34^+/CD110^+/CD45RA^-$), erythroid progenitors (Ery, $CD34^+/CD110^+/CD45RA^-/CD41^-$) and megakaryocytic progenitors (Mk, $CD34^+/CD110^+/CD45RA^-/CD41^+$). Using these sorting procedures we ware able to show that downmodulation of STAT5 in common myeloid, erythroid or megakaryocytic progenitors results in an increase in megakaryocyte progenitor numbers while changes in the GMP compartment were not detected.

Thus our data show that STAT5 determined lineage commitment between erythroid and megakaryocytic cell fates. Downmodulation of STAT5 reduced BFU-E and increased CFU-MKs while the numbers of myeloid progenitors were much less affected. Furthermore, activation of

STAT5 resulted in the onset of erythropoiesis while megakaryopoiesis was impaired.

RAS mutations are frequent in human myeloid leukemia especially in acute myeloid leukemia (AML), chronic myelomonocytic leukemia (CMML), and juvenile myelomonocytic leukemia (JMML).[24;25] Still, their function in human hematopoietic stem and progenitor cells is not well investigated. In **chapter 5,** we explored the effects of $KRAS^{G12V}$ on human stem and progenitor cells and studied how oncogenic KRAS might contribute to leukemic transformation. Using a retroviral expression system $KRAS^{G12V}$ was stably introduced into human cord blood $CD34^{+}$ cells and after retroviral transduction MS5 bone marrow stromal cocultures were initiated. These studies demonstrated that overexpression of constitutively active KRAS induced a dramatic increase in cell expansion which coincided with increased early cobblestone formation, induction of monocytic differentiation and a strong reduction of LTC-IC frequencies. By week three, progenitors disappeared from the cocultures and KRAS cells terminally differentiated. Our observations were in line with previous publications reporting that overexpression of oncogenic RAS in various mouse models could induce a myeloproliferative disease, but not leukemia.[26-28] Furthermore, these studies suggested that KRAS mutations required additional mutations to induce a frank malignancy.[29-31] When the isoforms of RAS (HRAS, KRAS and NRAS) were studied, it became clear that all of them had the potential to induce myeloid leukemia and initiate a CMML or AML-like disease.[32]

In addition, our experiments with unsorted cells revealed that non-transduced cord blood cells had a proliferative advantage in the presence of

the KRAS-transduced cells. Conditioned medium was harvested from KRAS-transduced cocultures and the medium was added to freshly isolated cord blood $CD34^+$ cells. These experiments showed that the conditional medium by itself was capable to increase expansion and induce cobblestone formation, but not monocytic differentiation. In order to elucidate signal transduction pathways involved in KRAS-induced transformation Western blot analysis was performed from control and KRAS-transduced cord blood cells. These experiments showed an increase in the expression of phospho-ERK1/2, and phospho-p38 levels in KRAS-transduced cells but JNK phosphorylation was not changed. To inhibit the $KRAS^{G12V}$-induced phenotype, a MEK inhibitor (U0126) and a p38 inhibitor (SB203580) were used. These experiments revealed that proliferation and CAFC formation were predominantly mediated by ERK and that the differentiation process was mediated by the p38 signaling route.

To gain more insight into mechanisms by which oncogenic $KRAS^{G12V}$ contributes to the process of leukemic transformation, we performed multiplex cytokine arrays and genome-wide gene expression profiling. Cytokines that were highly expressed are CCL2, IL1RA, and interleukin-8. These findings are in line with a recently published study where different human cell lines expressing $KRAS^{G12V}$ or activation of RAS/ERK signaling showed increased expression of growth factors and cytokines for example VEGF, IL6, CXCL1, or interleukin-8.[33-35] Our data also show that the increased proliferation in KRAS-activated cells is associated with increased expression levels of the cell cycle regulators CYCLIN D1, CYCLIN D2, and CYCLIN D3. Furthermore, it has been reported that interleukin-8 promotes tumor cell proliferation and induces cell migration in colon cancer cells.[36-39] Interleukin-8 is an initiator of the

chemotactic migration of leucocytes and it might be that KRAS-induced interleukin-8 secretion induces migration towards the RAS-activated cell. However, this hypothesis still needs to be confirmed.

In summary, we found that overexpression of $KRAS^{G12V}$ in human stem and progenitor cells transiently increases expansion and early cobblestone formation, promotes myelo-monocytic differentiation and reduces LTC-IC frequencies. Our data show that $KRAS^{G12V}$ activates both ERK and p38MAPK pathways, but not JNK in $CD34^+$ CB cells. Activation of the ERK pathway induces proliferation which is mediated at least in part via secreted factors that can act in an autocrine/paracrine manner.

The development of acute myeloide leukemia is a multistep process in which several transformation events occur such as chromosomal translocations, point mutations, insertions and epigenetic alterations. It is likely that a number of sequential events co-operate, which cause enhanced self-renewal and abnormalities in differentiation, and lead to the development of leukemic stem cells.[40-42] The observation that the incidence of AML is increased in elderly people further strengthens the idea that accumulation of genetic and/or epigenetic events play a role in the development of leukemia. Thus, understanding the mechanisms that are involved in self-renewal and differentiation in the hematopoietic system could contribute to our understanding how AML develops.

Currently there are two basic approaches to study leukemia. One of them is to study primary leukemic cells as a starting point. The other approach uses healthy hematopoietic stem/progenitor cells in which (epi)genetic mutations are introduced in order to model leukemic transformation. For both approaches, the use of (xeno)transplant model

systems is required to demonstrate in vivo the development of leukemia. These models have been developed and greatly improved over the last decade, which has resulted in the identification of cancer stem cells that initiate and sustain leukemic proliferation, and has firmly established the cancer stem cell hypothesis.[43-45]

While the use of primary human patient samples as a starting point has allowed the identification of cells that can maintain the leukemia upon transplantation in xenotransplantation models, it is difficult to retrospectively trace back which and when (epi)genetic mutations occurred during the leukemic transformation process. Over the years, it has become clear that leukemogenesis is a multistep process whereby a number of (epi)genetic alterations accumulate over time, and studying primary leukemic cells at the moment at which the disease has profoundly manifested itself does not give much insight into the steps which have lead to the development of the LSC. To study the involvement of the individual genetic alterations in the development of leukemia, a different approach was necessary. Introduction of single or multiple genetic alterations in mouse models or primary human hematopoietic cells has helped tremendously to understand the role of certain genes in hematopoiesis and in the development of AML.[46-53] Using these model systems it was realised that a single genetic alteration is not enough in most of the cases to induce leukemia.

The work presented in this thesis contributes to further understanding the role of two genes, STAT5 and KRAS in the development of AML. We overexpressed STAT5 and KRAS in primary hematopoietic cells and investigated their contribution to leukemic transformation. We discussed

mechanisms that could lead to leukemic transformation. Identification of these novel mechanisms can lead to better understanding of the development of acute myeloid leukemia and might eventually help to improve treatment outcome.

Future perspectives

Leukemic transformation is a multistep process which involves a number of events such as chromosomal translocations, point mutations, insertions and aberrant epigenetic regulation. The studies in this thesis aimed to further elucidate the role of STAT5 and KRAS in the development of acute myeloid leukemia and investigate how they contribute to leukemic transformation.

During our investigations we observed that overexpression of oncogenic KRAS induces a strong differentiation program towards the monocytic lineage. The oncogenic KRAS-induced differentiation coincided with reduction of total CFC numbers and LTC-IC frequencies. These observations were in agreement with other published data where oncogenic RAS was not sufficient to induce frank malignancy.[30;31] These findings suggest that KRAS requires co-operating events as a second hit to overcome the strong KRAS-induced monocytic differentiation program by introducing a differentiation block. Alternatively, KRAS itself might be the second hit which favours differentiation into the monocytic lineage after a mutation in another gene caused high self-renewal. Future research should be directed at investigating genes which could mediate these phenomena in AML.

The polycomb group protein, BMI1, has been shown to induce HSC self-renewal and long-term stem cell maintenance. Overexpression of BMI1 enhanced symmetric cell divisions of HSCs and consequently led to increased HSC self-renewal.[54-56] Because of these properties overexpression of BMI1 might be a good strategy to increase self-renewal and induce immortalisation in the oncogenic KRAS-transduced cells.

In chronic myelomonocytic leukemia TET2 mutations are also frequently demonstrated.[57] Cells with TET2 mutation from patient with myeloproliferative disorder showed a better hematopoietic reconstitution in NOD/SCID mice than cells without TET2 mutation. Bone marrow samples were analyzed from NOD/SCID mice after 15 weeks of transplantation and TET2 mutations were found in all long-term-culture-initiating cells and progenitors.[58] Since both KRAS mutations and TET2 mutations frequently occur in chronic myelomonocytic leukaemia it would be intriguing to further investigate the role of the TET2 mutation in KRAS activated cells and introduce TET2 mutation as an additional event.

In normal hematopoiesis the transcription factor CEBPα is essential for the CMP-GMP transition.[59] Furthermore, 9% of AML cases show mutations in the CEBPα gene. In leukemic cells two types of CEBPα mutations can be observed; N-terminal mutations and the C-terminal mutations. Mice modelling the N-terminal CEBPα mutations developed AML and the committed myeloid cells from these animals were able to transfer the disease to secondary recipients indicating that the N-terminal CEBPα mutations are capable to impose self-renewal.[60] Using knockin mouse models it was demonstrated that C-terminal CEBPα mutations increase proliferation of HSCs and mice develop leukemia with immature

erythroid phenotype.[61] Because of the phenotypes mentioned above it would be appealing to study whether introduction of CEBPα mutations in KRAS transduced cells could delay the KRAS induced differentiation program and induce long-term growth.

Another approach could be to block myeloid differentiation by inhibiting PU.1 with RNAi in oncogenic KRAS-transduced cells. PU.1 is a key transcription factor in myeloid development and it is curtail in normal myeloid differentiation. New born $PU.1^{-/-}$ mice have no detectable monocytes/macrophages or neutrophil granulocytes.[62;63] These data suggest that downmodulation of PU.1 could introduce a differentiation block in oncogenic KRAS transduced cells and possibly could extend the lifetime of the double transduced cells.

Genome-wide gene expression profiling showed that STAT5 target genes in HSC, CMP, GMP and MEP compartments were different and the numbers of genes which were commonly expressed in the HSC and progenitor compartments were relatively small. This data suggests that STAT5 cofactors, repressors and transcriptional regulators are tissue-specific. Because of these reasons it would be intriguing to further investigate the mechanisms which influence gene regulation in stem and progenitor compartments.

An interesting candidate to study could be PIM1. We observed that PIM1 was significantly overexpressed in HSC, CMP and MEP but not in GMP. The human *PIM1* gene encodes a serine/threonine kinase which is a downstream effector of many cytokine-signaling pathways. Furthermore PIM1 has been shown to be involved in transcriptional regulation, cell cycle

regulation and survival.[64;65] One of the possibilities why PIM1 is not upregulated in the GMP compartment is that in GMPs other cofactors are expressed than in HSCs, CMPs and MEPs or that STAT5 recruited cofactors in GMP have different binding potential. Furthermore it is also possible that the various STAT5 complexes have various mechanisms to regulate gene transcription. To investigate this it would be informative to perform STAT5 chromatin immunoprecipitation in control and STAT5-activated HSC and progenitor cells in order to determine whether promoter occupancy is different in these hematopoietic compartments.

STAT5 activation induced expression of HIF2α in cord blood $CD34^+$ cells. Furthermore, downmodulation of HIF2α resulted in decrease of expansion, CFC numbers and LTC-IC frequencies. Until now little is known about HIF2α target genes and microarray analysis on HIF2α–overexpressing cord blood cells and/or on STAT5-activated cells in which HIF2α is downmodulated could give more insight into the mechanisms by which HIF2α contributes to the STAT5-induced phenotypes. Although the involved mechanisms are currently unclear, it has been suggested that EGFR and IGFR1 can be activated by HIF2α, which might contribute to the activation of AKT and ERK signalling, thus inducing proliferation.[11] The involvement of these pathways in the STAT5-HIF2α-induced proliferation could be studied as well.

While our studies indicated that HIF2α is required downstream of STAT5 to increase proliferation, CFC numbers and LTC-IC frequencies, it is currently unclear whether expression of HIF2α by itself would be sufficient to induce these phenotypes. By making use of e.g. stabilized mutants of HIF2α that are insensitive to VHL-induced hydroxylation and

degradation under normoxic conditions this question can be addressed. It was reported previously that HIF protein levels were not only upregulated in various malignancies but they were also stabilized. Based on these observations we measured HIF2α protein levels in AML samples by Western blot. Even though till now relatively few samples have been analyzed, preliminary data show that about half of the investigated AML cases express higher levels of HIF2α protein as compared to $CD34^+$ cells from peripheral blood or cord blood. Furthermore, it would be intriguing to downmodulate HIF2α in these primary AML cells to determine whether these elevated HIF2α levels are indeed required to maintain the leukemic phenotype.

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Chapter 7

Summary in Dutch (Nederlandse samenvatting)

Nederlandse samenvatting

Bloed is een gespecialiseerd weefsel dat zorgt voor toevoer van noodzakelijke voedingsstoffen en zuurstof, afvalproducten van cellen afvoert en bijdraagt aan het immuunsysteem tegen ziekteverwekkers. Het menselijk bloed bestaat uit bloedcellen en vloeibaar plasma. Bloedcellen kunnen worden onderverdeeld in rode bloedcellen die rood hemoglobine bevatten en zuurstof transporteren, en witte bloedcellen die betrokken zijn bij de bescherming van het lichaam tegen infecties en parasieten. Alle bloedcellen in ons lichaam komen voort uit bloedstamcellen die gelokaliseerd zijn in het beenmerg. Bloedstamcellen kunnen veranderen (differentiëren) in voorlopercellen die uiteindelijk de rijpe bloedcellen vormen, maar kunnen ook zichzelf vernieuwen waardoor er altijd een hoeveelheid bloedstamcellen aanwezig is in het beenmerg. Hierdoor blijft de bloedproductie tijdens ons leven gegarandeerd. De balans tussen zelfvernieuwing en differentiatie is sterk gereguleerd en vele bloedziekten, waaronder acute myeloide leukemie, kennen hun oorsprong in een verstoring van deze balans.

De ontwikkeling van acute myeloide leukemie is een meerstaps proces waarin de cel verschillende veranderingen ondergaat. Het ligt voor de hand dat een aantal van deze veranderingen gezamenlijk bijdragen aan toegenomen zelfvernieuwing, afwijkingen in differentiatie en leiden tot de ontwikkeling van leukemische stamcellen.

De laatste jaren is het duidelijk geworden dat kankercellen in acute myeloide leukemie niet allemaal dezelfde karakteristieken bezitten maar van elkaar verschillen en een hiërarchische structuur ten opzichte van elkaar

hebben. Aan de top van de hiërarchie staan de leukemische stamcellen die kunnen zelfvernieuwen. Leukemische stamcellen vermeerderen zich niet alleen, maar vormen ook andere kwaadaardige cellen die hun mogelijkheid tot zelfvernieuwing verliezen. Deze cellen kunnen verder groeien en differentiëren, maar vormen geen normale functionele bloedcellen. De grote hoeveelheid van deze niet-functionele kankercellen verstoort de normale bloedvorming wat leidt tot bloedarmoede, bloedingen en een slecht functionerend immuunsysteem. Daarnaast kunnen leukemische stamcellen zich buiten het bloed in andere delen van het lichaam verspreiden, waaronder het centraal zenuwstelsel, de huid en het tandvlees wat leidt tot verdere complicaties.

Chromosomale translocaties en moleculaire afwijkingen in stamcellen verstoren het normale proces van zelfvernieuwing en differentiatie wat kan leiden tot de ontwikkeling van bloedkanker. Er zijn een aantal belangrijke moleculen geïdentificeerd die niet correct functioneren in leukemische cellen. In dit proefschrift onderzochten we de rol van STAT5 en KRAS in zelfvernieuwing en differentiatie van bloedstamcellen en hun potentiële rol in de ontwikkeling van leukemie.

STAT5 is een transcriptiefactor die de expressie reguleert van een aantal genen in de cel. STAT5 is aanwezig in veel bloedcellen en speelt een essentiële rol bij de normale bloedvorming (hematopoïese). Meer dan 65% van de patiënten met acute myeloide leukemie heeft een constitutief geactiveerd STAT5, wat bijdraagt aan de ontwikkeling van acute myeloide leukemie. Een van de eiwitten die verstoord tot expressie komt onder invloed van constitutieve activatie van STAT5 is het membraaneiwit MUCIN1. In **hoofdstuk 2** onderzochten we de rol van MUCIN1 in de

normale bloedvorming en acute myeloide leukemie. In materiaal van acute myeloide leukemie patiënten vonden we dat MUCIN1 verhoogd tot expressie komt in 70% van de gevallen, wat suggereert dat dit bijdraagt aan de ontwikkeling van de ziekte. Door middel van stabiele overexpressie van MUCIN1 in gezonde bloedcellen hebben we aangetoond dat MUCIN overexpressie leidt tot een verhoogd aantal celdelingen, een toegenomen aantal stam- en voorlopercellen, en een versterkte interactie tussen bloedcellen en beenmergcellen die de bloedvorming ondersteunen.

In **hoofdstuk 3** bestudeerden we of STAT5 activatie specifiek de lange-termijn groei van stamcellen induceert, of ook de groei van voorlopercellen beïnvloedt. Deze experimenten lieten zien dat STAT5 activatie alleen de groei van stamcellen, maar niet die van voorlopercellen, stimuleert wat suggereert dat STAT5-geïnduceerde lange-termijn groei de basis vindt in de stamcellen en niet de voorlopercellen.
Vervolgens hebben we genen geïdentificeerd die gereguleerd worden door STAT5 in de stamcelpopulatie. Een van deze genen was hypoxia-induced factor 2 (HIF2). Wanneer we de expressie van HIF2 artificieel verlagen in STAT5-geactiveerde cellen zagen we verminderde proliferatie, en een verlaagd aantal stam- en voorlopercellen. Deze data laten zien dat STAT5-gemedieerde lange-termijn effecten (gedeeltelijk) worden gereguleerd door HIF2, en dat STAT5 activatie de expressie van hypoxie-geïnduceerde genen veranderd door middel van HIF2 toename. Kankercellen kunnen wellicht met behulp van dit mechanisme hun ongedifferentieerde eigenschappen bewaren.

In **hoofdstuk 4** onderzochten we de potentiële rol van STAT5 in de ontwikkeling van erythrocyten (rode bloedcellen) en megakaryocyten (productie bloedplaatjes). Onze resultaten laten zien dat verlaging van STAT5 expressie resulteert in toegenomen aantalen megakaryocytaire voorlopercellen, terwijl erythroide voorlopercellen verminderd waren. Overexpressie van een constitutief actieve vorm van STAT5 - STAT5A(1*6)- laat echter een verminderde megakaryocytaire ontwikkeling zien en een robuuste inductie van erythroide differentiatie. Deze data laten zien dat STAT5 activatie erythroide differentiatie induceert terwijl de megakaryocytaire ontwikkeling verminderd is. Daarnaast blijkt dat naast de effecten op het stamcelcompartiment, zoals beschreven in hoofdstuk 3, STAT5 ook betrokken is bij differentiatie beslissingen van voorlopercellen.

RAS mutaties komen frequent voor bij acute myeloide leukemie. Echter, hun functie in humane hematopoïetische stam- en voorlopercellen is niet goed begrepen. In **hoofdstuk 5**, onderzochten we de effecten van oncogeen KRAS op humane stam- en voorlopercellen en bestudeerden hoe KRAS bijdraagt aan leukemische transformatie. Onze resultaten demonstreren dat overexpressie van oncogeen KRAS in bloedcellen leidt tot een dramatische toename in celgroei die samengaat met toegenomen vroege cobblestone vorming, inductie van monocytaire differentiatie en een sterke reductie in stamcelfrequentie. Daarnaast laten onze experimenten zien dat oncogeen KRAS de ERK en p38MAPK signaaltransductieroutes activeert. Differentiatie wordt voornamelijk door p38 gemedieerd, terwijl de ERK proliferatie stimuleert, in ieder geval gedeeltelijk gereguleerd door gesecreteerde factoren. Deze studies laten zien dat KRAS mutaties op

zichzelf niet voldoende zijn om leukemie te veroorzaken maar dat additionele mutaties nodig zijn.

Het werk beschreven in dit proefschrift draagt bij aan een toegenomen begrip van de rol van STAT5 en KRAS in de ontwikkeling van acute myeloide leukemie. Door middel van overexpressie van STAT5 en KRAS in primaire bloedcellen onderzochten we hun bijdrage aan leukemische transformatie. Verder discussieerden we over de mechanismen die zouden kunnen leiden tot leukemische transformatie. Identificatie van deze nieuwe mechanismen kan leiden tot een verbeterd begrip van de ontwikkeling van acute myeloide leukemie en verbetering van de behandeling.

List of abbreviations

AML	acute myeloid leukemia
AP1	activator protein 1
APL	acute promyelocytic leukemia
ATF	activating transcription factor
BM	bone marrow
C/EBPα	CCAAT/enhancer binding protein, alpha
CB	cord blood
CCL	chemokine (C-C motif) ligand
CFC	colony forming cell
CLP	common lymphoid progenitor
CML	chronic myeloid leukemia
CMML	chronic myelomonocytic leukemia
CMP	common myeloid progenitor
CMV	cytomegalovirus
CT	cytoplasmic tail
CXCR	chemokine (C-X-C motif) receptor
EPO	erythropoietin
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FLT3	fms-like tyrosine kinase 3
G-CSF	granulocyte colony-stimulating factor
GDP	guanine diphosphate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte–macrophage progenitor
GRB2	growth factor receptor bound protein 2
GSK3	glycogen synthase kinase 3
GTP	guanine triphosphate
HIF	hypoxia-inducible factors
HOX	homeobox
HSC	hematopoietic stem cell
ICAM	inter-cellular adhesion molecule
IκB	inhibitor κB

IKK	IκB kinase
IL	interleukin
ITD	internal tandem duplication
JAK	janus kinase
JMML	juvenile myelomonocytic leukemia
JMML	juvenile myelomonocytic leukemia
JNK	c-jun N-terminal kinase
LIC	leukemia-initiating cell
LSC	leukemic stem cell
LTC-IC	long-term culture-initiating cell
LT-HSC	long-term hematopoietic stem cell
MAPK	mitogen-activated protein kinase
MDS	myelodysplastic syndrome
MEK	MAPK/ERK kinase
MEP	megakaryocyte-erythroid progenitor
MLL	multilineage leukemia
MPD	myeloproliferative disease
MPP	multipotent progenitor
NF-κB	nuclear factor kappa B
NOD/SCID	nonobese diabetic/severe combined immunodeficiency
NPM	nucleophosmin
PI3-K	phosphatidylinositol 3-kinase
PIAS	protein inhibitor of activated STAT
PTH	parathyroid hormone
Q-PCR	quantitative PCR
ROS	reactive oxygen species
SCF	stem cell factor
SDF	stromal cell-derived factor
SH2	Src homology domain
SOCS	supressor of ctokine sygnaling
STAT	signal transducer and actvator of transcription
ST-HSC	short-term hematopoietic stem cell
TPO	thrombopoietin
VEGF	vascular endothelial growth factor
WT	wild-type

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Hematopoietic stem cells but not progenitors are the exclusive target for STAT5 induced long-term self-renewal.
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